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ARCHITECTURE AND INTERACTIONS OF THE *Saccharomyces cerevisiae* ELONGATOR COMPLEX

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by

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ABSTRACT

Yeast Elongator was initially isolated based on its interaction with the hyper-phosphorylated form of RNA polymerase II. Later on, it was shown to possess an intrinsic histone acetyl-transferase activity and to crosslink to nascent RNA *in vivo*. The six *ELP* genes were also identified, among other genes, in a genetic screen in *Saccharomyces cerevisiae* for targets of the toxin zymocin. *KTI12*, which stands for "*K.* lactis toxin insensitive 12", was one of those other genes. *kti12Δ* mutant cells display phenotypes closely resembling those of *elpΔ* mutants. Moreover, Kti12 protein was shown to co-immunoprecipitate with Elongator and with RNA polymerase II, indicating a functional interaction with both factors.

The experiments presented in the first part of this thesis confirm genetically and biochemically that Elongator is a six-subunit complex. *In vitro* and *in vivo* studies were performed to reveal the molecular architecture of this complex. Briefly, strong pair-wise interactions between Elp1 and Elp3, Elp4 and Elp6 and between Elp5 and Elp6 were uncovered. Additionally, a weak interaction between Elp3 and Elp4 was observed. *In vitro* HAT assays and RNA immunoprecipitation experiments suggested that Elp2 is dispensable for the *in vitro* histone acetyl-transferase and the *in vivo* RNA binding activity of Elongator. In contrast, Elp3 was shown to be critical for both the integrity of the complex and its *in vivo* RNA binding activity. Moreover, the localisation of yeast Elp3 protein was studied, in an attempt to address the possibility that Elongator continuously shuttles from the cytoplasm to the nucleus.

In the second part, biochemical and genetic studies strongly suggested that Kti12 interacts with Elongator and might regulate its *in vivo* HAT activity. Finally, the molecular mechanism of action of the toxin zymocin was studied. In particular, preliminary experiments, which test the possibility that the RNAPII gets degraded in response to zymocin treatment of *S. cerevisiae* cells, are presented.

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ABBREVIATIONS AND NOMENCLATURE

Ade	Adenine
C. elegans	*Caenorhabditis elegans*
ChIP	Chromatin immunoprecipitation
CTD	C-terminal Domain
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	deoxy-NTP Nucleoside-5'-triphosphate
E. coli	*Escherichia coli*
Elp	Elongator protein
GENE1	Wild type *S. cerevisiae* gene
gene1	*S. cerevisiae* gene with a loss of function mutation
GFP	Green fluorescent protein
GTF	General Transcription Factor
HA	Haemaglutin
HAT	Histone Acetyl-Transferase
HDAC	Histone De-acetylase
His	Histidine
Kan	Kanamycin
kDa	kiloDalton
k/o	knock out
Leu	Leucine
LMB	Leptomycin B
Lys	Lysine
MAT	Yeast Mating type
mM	milli Molar
mRNA	messenger RNA

NEB	New England Biolabs
NES	Nuclear export signal
ORF	open reading frame
Pol II	RNA polymerase II
RIP	RNA immunoprecipitation
RNA	Ribonucleic acid
RNAse	Ribonuclease
RNAPII	RNA Polymerase II
rpm	revolutions per minute
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium Dodecyl Sulphate
SDS PAGE	Sodiun dodecyl sulphate polyacrylamide gel electrophoresis
TAF	TBP Associated Protein
TBP	TATA Binding Protein
Trp	Tryptophan
ts	Temperature sensitive
Ura	Uracil
WCE	Whole cell extracts

CHAPTER 1

INTRODUCTION

1.1 *The* RNA Polymerase II *transcription cycle*

1.1.1 Transcriptional activation

Expression of protein-coding genes in eukaryotic organisms is regulated in a highly orchestrated and elaborate fashion to ensure that specific genes are turned on and off in a temporally and spatially appropriate manner according to genetic blueprints, cell cycle requirements, environmental factors and, in metazoans, tissue-restrictive controls.

Over the last two decades, intensive efforts have succeeded in identifying the cellular components mediating the regulation of mRNA synthesis in eukaryotes. These polypeptides were initially isolated using biochemical purification strategies by tracking activities in reconstituted *in vitro* assays (Reese, 2003). Using such assays, core RNAPII was found to require additional biochemically defined factors, referred to as general transcription factors (GTFs), in order to perform promoter-specific transcription. Experiments performed in the early 1980s demonstrated that RNAPII and GTFs are sufficient to mediate basal transcription (Matsui et al., 1980). However, high levels of regulated transcriptional activity additionally required the action of sequence-specific activators that bind to cognate DNA elements. Conversely, transcription can be inhibited by gene-selective transcriptional repressors, which when bound to repressive DNA elements prevent the transcriptional machinery from transcribing a gene (Wolffe et al., 1997).

Very early on, it was shown that transcriptional activators posses modular domains that mediate binding to DNA and transactivation functions, respectively (Mitchell and Tjian, 1989). The activation domains were initially thought to directly recruit

components of the transcription machinery to the promoters (Mitchell and Tjian, 1989). However, the identification of factors, which interact with and facilitate or mediate the action of sequence-specific regulators, led to critical re-evaluation of the early models. These factors were named co-activators (Berger et al., 1990; Chen and Evans, 1995; Dynlacht et al., 1991; Kelleher et al., 1990; Kim et al., 1994b; Meisterernst and Roeder, 1991; Pugh and Tjian, 1990). The recent finding that many of the co-activators harbor chromatin directed activities, such as histone modifier complexes and chromatin remodeling complexes, added another level of complexity. Now it is well established that transcriptional co-activators can be classified into two categories: (i) co-activators that interact with or are themselves components of the RNA Polymerase II machinery, such as TFIID and Mediator and (ii) chromatin-directed co-activators, such chromatin remodeling complexes and histone modifiers, which prepare the nucleosomal environment for binding of other components of the transcription machinery (Naar et al., 2001). According to the current model there is a synergistic effect on transcriptional activation by multiple copies of the same activator or even different activators, which recruit different co-activator complexes on the same promoter. This allows gene-specific and often highly sensitive transcriptional responses to cellular signals and small changes in activator abundance (Naar et al., 2001).

A number of recent studies, utilizing the chromatin immunoprecipitation assay, have shed light on the order of events, which lead to transcriptional activation of a number of different genes (Cosma, 2002). According to results from work with model-genes, the activators can be classified into two categories: those that

bind to nucleosomal templates and are able to perturb chromatin structure, such as Pho4, estrogen receptor and Swi5 (Cosma et al., 1999; Reinke et al., 2001; Shang et al., 2000) and those, which bind to promoter regions only after nucleosomes have been conformationally changed, such as SBF and E2Fs (E2F1, E2F2, E2F3) (Cosma et al., 1999; Rayman et al., 2002). The latter need chromatin-modifying activity to precede their binding.

Both activators and co-activators facilitate the recruitment and assembly of the components of the pre-initiation complex. The next steps in transcription initiation include open complex formation, promoter melting and finally formation of the first phosphodiester bond (initiation). Then, when the actual process of transcription has begun, polymerase needs to escape from the promoter and become engaged in processive mRNA production (promoter clearance, early elongation). A transcription elongation complex, composed of the elongating form of RNA Polymerase II and its co-factors (different from the ones that are needed for initiation), is now formed, and travels along the coding region of the gene (transcript elongation). As transcript elongation proceeds, the RNA matures by co-transcriptional capping, splicing, and poly-adenylation. Finally, transcription terminates and the mature RNA is ready to be transferred to the cytoplasm for translation. Factors required for RNA export are also believed to associate with RNAPII and the nascent transcript already during transcript elongation (Svejstrup, 2004). Through all this, it should be remembered that not only initiation, but also subsequent events occur in the context of chromatin.

Next, I will first describe the mechanism of transcription

initiation and then the co-activators, which function prior to or during this process.

1.1.2 The mechanism of transcription initiation

The mechanism of transcription initiation can be subdivided in three steps: pre-initiation complex assembly, open complex formation and finally, promoter melting and initiation. *In vitro* and *in vivo* data have suggested that there are two models for pre-initiation complex assembly: the stepwise and the holo-enzyme model (Svejstrup, 2004). The "stepwise" model is characterized by a distinct series of events:

1. Recognition of core promoter by TFIID (TBP and associated factors)

TFIID is composed of TBP (TATA-binding protein) and at least 14 tightly associated factors, the TBP associated factors (TAFIIs) (Albright and Tjian, 2000). Numerous studies have established that this multi-subunit complex is responsible for recognition of the TATA element, a process which involves DNA distortion (Kim et al., 1993a; Kim et al., 1993b; Tsai and Sigler, 2000). More recent structural evidence show that TBP is responsible for bending the TATA box DNA, creating a context for the binding of TFIIB, and a curvature in the DNA appropriate for wrapping around RNA Polymerase II (Bushnell et al., 2004). The binding of TFIID on the TATA element is believed to act as a platform for the nucleation of the remaining factors of the pre-initiation complex. However, there are promoters, which lack a canonical TATA box. These can be recognized by TBP-like factors (TLFs), or other complexes, which include TAFIIs but do not contain TBP or TBP-like factors (Juo et al., 1996; Rabenstein et al.,

1999; Wieczorek et al., 1998). Finally, it should be mentioned that it is now generally believed that, apart from TBP or TLFs, TAFIIs also play an important role for DNA sequence recognition (Burke and Kadonaga, 1997; Wu et al., 2001).

2. Recognition of TFIID-promoter complex by TFIIB

The binding of TFIIB to the TFIID-promoter complex results in a more stable ternary complex. Early studies in yeast demonstrated that TFIIB interacts with RNA Polymerase II and that this interaction is crucial for specifying the start site of transcription (Li et al., 1994; Pinto et al., 1992). TFIIB contains two functional domains. The conserved C-terminus interacts with TBP (Buratowski et al., 2002). Both human and yeast TFIIBc have been shown to bind upstream and downstream of the TATA box (Bushnell et al., 2004; Tsai and Sigler, 2000). The N-terminus binds to the RNA Polymerase II/TFIIF assembly and brings it into the pre-initiation complex (Bushnell et al., 2004; Pardee et al., 1998). Structural studies have recently suggested that interactions of TFIIB with both TBP and with RNAPII determine the location of the transcription start site (Bushnell et al., 2004). The C- and N-terminal domains of TFIIB are separated by a flexible linker, which is believed to act as a molecular switch, regulating changes in the conformation of TFIIB. This conformation change may affect start-site selection, promoter recognition and transcription activation (Fairley et al., 2002).

3. Recruitment of a TFIIF/RNAPII complex

Once TFIID and TFIIB have assembled on the promoter, RNA Polymerase II can enter the pre-initiation complex. However,

polymerase cannot stably associate with this GTFs sub-assembly and must be escorted to the promoter by TFIIF. TFIIF is composed of two subunits and was shown very early on to reduce the non-specific DNA affinity of RNAPII *in vitro*, thereby ensuring that polymerase only enters productive complexes at a TATA-box (Finkelstein et al., 1992). This function of TFIIF was also suggested by more recent photo-cross-linking studies, where it was shown to locate both upstream and downstream of the transcription start site (Forget et al., 2004), all in all allowing the proposition that the recognition of the TFIID-TFIIA-TFIIB complex at the TATA box by an RNAPII-TFIIF complex might be an early "checkpoint" for correct promoter complex assembly (Svejstrup, 2004). TFIIF has been shown to have other roles during the transcription cycle; stabilization of binding of RNA Polymerase II to TFIID and TFIIB at the promoter, facilitation of the entry of TFIIE and TFIIH (Buratowski et al., 1991; Flores et al., 1992), contribution to open complex formation by the DNA helicase activity of TFIIH (Conaway and Conaway, 1993) and to synthesis of the first phosphodiester bond of nascent transcripts (Tirode et al., 1999). Moreover, as will be discussed later, TFIIF may also play a role during transcript elongation. Finally, after transcription termination, TFIIF is thought to recruit and stimulate the activity of the TFIIF-associating CTD phosphatase (FCP1) for recycling of the hyper-phosphorylated form of RNA Polymerase II (IIO) to the un-phosphorylated one (IIA), before the next round of transcription initiation (Chambers et al., 1995; Friedl et al., 2003; Kamada et al., 2003).

4. Binding of TFIIE and TFIIH

It is widely accepted that TFIIE and TFIIH stabilize and activate PIC formation by binding to all the other general transcription factors as well as to RNA Polymerase II, and at the same time open up the double stranded DNA (dsDNA) at the region from -9 to +2, relative to the transcription initiation site (+1), in a manner that is dependent on ATP hydrolysis (Douziech et al., 2000; Dvir et al., 1997; Holstege et al., 1996; Leuther et al., 1996; Robert et al., 1996; Yamamoto et al., 2001). After promoter melting, transcriptional initiation occurs by the incorporation of nucleotide triphosphates into a nascent RNA chain. TFIIE and TFIIH both appear to play important roles in the subsequent transition from initiation to elongation, a process known as promoter clearance (Goodrich and Tjian, 1994; Kumar et al., 1998; Yamamoto et al., 2001).

The function of TFIIE is closely related to that of TFIIH. In particular, TFIIE facilitates the recruitment of TFIIH to the PIC, and stimulates TFIIH-dependent ATP hydrolysis. The latter is necessary for promoter melting and TFIIH-dependent phopshorylation of the RNAPII CTD, which is required for efficient promoter clearance (Ohkuma, 1997; Watanabe et al., 2000). Moreover, TFIIE has been proposed to be the direct target of certain gene-specific transcriptional activators (Sauer et al., 1995; Zhu and Kuziora, 1996). Such interactions are needed for TFIIE recruitment to the PIC.

TFIIH, on the other hand, is a nine-subunit complex (Svejstrup et al., 1994). TFIIH can be disrupted biochemically into a six-subunit core-TFIIH, which forms part of a larger assembly (or "repairosome") that functions in NER (nucleotide excision repair) (Svejstrup et al.,

1995), and a three-subunit TFIIK complex, which has CTD kinase activity (Feaver et al., 1994; Svejstrup et al., 1994; Takagi et al., 2003). The major contributions of TFIIH in RNAPII transcription is in ATP-dependent promoter melting (Douziech et al., 2000) and the regulation of the transition from initiation to elongation by phosphorylating the C-terminal repeat domain (CTD) of Rpb1, the largest RNAPII subunit. Kin28/CDK7 is the kinase responsible for this phosphorylation (Feaver et al., 1994). Consistent with this scenario, recent ChIP experiments show a dependence of CTD Ser5 phosphorylation on the presence of active TFIIH (Komarnitsky et al., 2000). Moreover, TFIIH was shown to promote the transition of very early elongation complexes to stable elongation complexes *in vitro* (Dvir et al., 1997). Thus, TFIIH is believed to perform multiple roles, affecting steps before, during and immediately after initiation of transcription. TFIIH may also affect transcript elongation.

5. Recruitment of TFIIA

TFIIA can be recruited at any stage after the binding of TFIID and stabilizes the initiation complex. The precise role of TFIIA in transcription initiation has been the subject of much controversy, because the requirement for TFIIA in reconstituted transcription varies from system to system. Early *in vitro* studies indicated that TFIIA was largely dispensable for activator-independent transcription (DeJong et al., 1995; Hansen and Tjian, 1995). However, more recent studies shed light on the essential role for TFIIA in transcription activation of many genes. TFIIA binds TBP opposite to the side where TBP dimerizes, and interacts with TFIIB (Coleman et al., 1999; Liu et al., 1999). Binding of TFIIA to auto-

repressed TBP dimers induces their dissociation and accelerates the kinetics of TBP-DNA binding (Coleman et al., 1999). The association of TFIIA with TBP/TFIID is inefficient and enhanced by transcriptional activators (Xing et al., 2001).

The holo-enzyme model

According to the model so far described for pre-initiation complex assembly, the GTFs exist separately in solution and come together only upon association with promoter DNA. However, some studies have reported that a subset of GTFs exist in a pre-assembled form, in an RNA Polymerase II "holo-enzyme", which has been proposed to bind to promoters in a single step. Ten years ago, two "holo-enzyme" complexes were purified from yeast and named SRB and Mediator complex (Kim et al., 1994a; Koleske and Young, 1994; Thompson et al., 1993; Wilson et al., 1996). It soon became apparent that they were the same complex, except that the holo-enzymes isolated by Young and co-workers also contained general transcription factors, while the Kornberg holo-enzyme did not. Since then, studies from both Young and Reinberg groups suggested the presence of holo-enzymes in mammalian cells as well. These enzymes contained RNAPII, a subset of GTFs and homologues of yeast Srb proteins (Chao et al., 1996; Maldonado et al., 1996).

However, more recently, the use of chromatin immunoprecipitation assays has shed more light on the process of transcription initiation. A number of genes in different organisms have been used as models to dissect the mechanism which underlies the activation of transcription (Cosma, 2002). Based on these more recent data, all the proteins are recruited to genes promoters in a stepwise,

ordered manner. There are several examples where RNAPII is recruited independently of GTFs (Agalioti et al., 2000), and even ones where RNAPII is recruited after the Mediator complex (Cosma et al., 1999), strongly supporting the "stepwise" model of transcription activation. In further support of this model, recent evidence from both *in vitro* and *in vivo* studies has also suggested that GTFs such as TFIID, TFIIA and TFIIB, as well as the Mediator complex, stay behind at the promoter when RNAPII engages in transcript elongation, allowing rapid entry of new polymerases for re-initiation of transcription at the gene (Yudkovsky et al., 2000). This finding is not consistent with the idea that pre-assembled holo-polymerase complexes play an important role for transcription.

1.1.3 The role of co-activators in transcription initiation

Mediator complex

The first indications of a common target for transcriptional activators came from the observation that one activator protein could interfere with the effects of another in an *in vitro* transcription system (Gill and Ptashne, 1988). This finding inspired the experiments that led to the discovery of co-activators and eventually to identification of a yeast Mediator complex which was able to reverse the interference and respond to activators in a pure *in vitro* transcription system (Flanagan et al., 1991; Kelleher et al., 1990; Kim et al., 1994b). Subsequently, yeast Mediator was purified both as a complex with RNAPII and as a discrete entity comprising 20 subunits, which was functionally defined by three activities: (1) stimulation of basal transcription in a highly purified system, (2) response to

transcriptional activators *in vitro* and *in vivo* and (3) stimulation of TFIIH-mediated phosphorylation of RNA Polymerase II (Kim et al., 1994b; Myers et al., 1998). To date, a number of mammalian complexes, which are thought to be the counterparts of yeast Mediator, have also been purified based on their ability to support the function of activators (Lemon and Tjian, 2000; Malik and Roeder, 2000).

The general requirement for individual Mediator subunits in global gene regulation differs from subunit to subunit. Some Mediator components are needed for the regulated expression of nearly all genes, whereas others are only required at a certain subset of genes (Holstege et al., 1998). Various biochemical experiments have suggested the existence of distinct sub-complexes of yeast Mediator. One of the more comprehensive approaches used urea to differentially dissociate yeast Mediator and to investigate the content of the distinct sub-complexes (Kang et al., 2001). These experiments identified two functionally distinct modules, called the Srb4 and the Rgr1 module, respectively. The Srb4 module interacts with the RNAPII CTD and other members of the general transcription machinery, such as TBP and TFIIB. The Rgr1 module comprises distinct Gal11 and Med9/10 sub-modules (Kang et al., 2001). Though the Med9/10 sub-complex interacts with the RNAPII transcription machinery, the Gal11 sub-module seems to be a major recognition unit for activators (Kang et al., 2001). Finally, the Srb8, -9, -10 and -11 may also constitute a distinct module. This module was recently purified as a separate entity from yeast extracts and its components have repressive functions in yeast (Borggrefe et al., 2002; Hengartner et al., 1998). Likewise, the human Srb10-Srb11 pair (CDK8-cyclin-C)

represses activator-dependent transcription *in vitro* and the large, transcriptionally inactive human ARC-L Mediator complex contains MED230, MED240, CDK8 and cyclin C, homologues of yeast Srb8, -9, -10 and -11, respectively (Akoulitchev et al., 2000; Taatjes et al., 2002).

A model has been proposed to explain the fate of Mediator during the transcription cycle (Svejstrup et al., 1997). According to this model, Mediator is recruited to gene-promoters *via* interaction with activators, binds to the un-phosphorylated form of RNAPII and stimulates CTD phosphorylation by TFIIH, which leads to promoter escape and transcript elongation. At this point, Mediator dissociates from phosphorylated RNA Polymerase II. After completing a round of transcription, the CTD is de-phosphorylated by CTD phosphatases. The un-phosphorylated Pol II can then enter a new cycle of transcription by again interacting with Mediator. Recently, Han and collaborators shed light on the re-initiation process of transcription (Yudkovsky et al., 2000). After transcription initiation, Mediator remains at the promoter and forms a scaffold with TFIID, TFIIA, TFIIH, and TFIIE that facilitates re-initiation. Interestingly, the stability of this scaffold is increased in the presence of activators.

Chromatin modifying complexes

Within a eukaryotic cell nucleus, genetic information is organized in a highly conserved structural polymer, termed chromatin. The fundamental repeating unit of chromatin is the nucleosome, which consists of 146 base pairs of DNA wrapped around an octamer of core histone proteins with two copies each of histone H2A, H2B, H3 and H4 (Kornberg, 1974). Linker histones of the H1 class and other

chromatin-bound proteins, such as high-mobility group (HMG) proteins, associate with DNA between single nucleosomes establishing the first higher level of organization, the so-called "solenoid" helical fibers (30 nm fibers) (Kornberg and Lorch, 1999). Chromatin architecture beyond these fibers is less clear, but folding and unfolding of putative super-structures are thought to have a pronounced impact on genomic function and gene activity (Fischle et al., 2003).

A number of studies have demonstrated that nucleosomal DNA is generally repressive to transcription (Grunstein, 1990). Therefore, an obvious question is how transcriptional activators and the core transcription machinery gain access to their cognate DNA binding sites and promoter elements in the restrictive environment of nucleosomal DNA. Part of the answer to this question was provided by the identification of dedicated chromatin modifying complexes, which serve to locally alter the structure of chromatin, facilitating gene-specific release from nucleosomal repression. Examples of such modifying complexes can be broadly grouped into two classes: (i) enzymes which covalently modify histones and (ii) ATP-dependent chromatin remodeling machines.

Core histone proteins are evolutionary conserved and consist mainly of flexible amino-terminal tails protruding outward from nucleosomes, and globular carboxy-terminal domains making up the nucleosome scaffold (Kornberg and Lorch, 1999). Histones, and mainly their N-terminal tails, function as acceptors for a variety of post-translational modifications, including acetylation, methylation and ubiquitination of lysine residues, phosphorylation of serine and threonine and methylation of arginine residues.

The subject of this thesis is very closely related to histone

modification and transcriptional regulation, since the yeast Elongator complex, the focus of this study, has been shown to posses histone acetyl-transferase (HAT) activity and to have roles in chromatin modification *in vivo* (Kristjuhan et al., 2002; Winkler et al., 2002). Moreover, sequence similarity analysis have been used to suggest that Elp3 might have a histone de-methylase activity as well (Chinenov, 2002). Therefore, it is relevant to here provide a short overview of the recent findings on histone modifications and transcriptional regulation.

Histone acetylation

One of the best-understood histone modifications is histone acetylation. It is now generally accepted that hyper-acetylation of histone tails, accomplished by histone acetyl-trasferase (HAT) enzymes, is mostly associated with activated, or euchromatic genomic regions, at both the local and global level. By contrast, de-acetylation, accomplished by histone de-acetylases (HDAC), mainly results in repression and silencing (heterochromatin) (Kuo and Allis, 1998).

Histone acetyl-transferases generally belong to one of two categories (Sterner and Berger, 2000): type A, located in the nucleus, which acetylate nucleosomal histones within chromatin and are potentially linked to transcription, and type B, mostly located in the cytoplasm, which are believed to have somewhat of a housekeeping role in the cell, acetylating newly synthesized, free histones. It should be mentioned that some of the A type enzymes also acetylate non-histone proteins (factor acetyl-trasnferases, FAT) (Imhof et al., 1997).

The first type A HAT identified was the *Tetrahymena* histone acetyl-transferase A (HAT A), which was found to be homologous to

Gcn5, a genetically defined transcriptional co-activator in yeast (Brownell et al., 1996). Since then, all the Gcn5-related HATs, one of which is the Elongator complex, have been grouped in the superfamily of GNAT (Gcn5-related N-acetyl-tranferase) enzymes. Another group of evolutionary related proteins that are known to be HATs belong to the MYST family, named for its founding members: human MOZ, yeast Ybf2/Sas3, Sas2, and human Tip60 protein (Borrow et al., 1996). Finally, a number of previously characterized transcriptional co-activators were shown to also posses HAT activity. This group includes the RNAPII-related human co-activators p300/CBP, SRC-1, ACTR, TIF2, and the TAFII250 subunit from various organisms (Chen et al., 1997; Mizzen et al., 1996; Ogryzko et al., 1996; Spencer et al., 1997).

The majority of the above mentioned HATs are found in multi-subunit complexes, which are recruited to gene promoters *via* an interaction with activators (Carrozza et al., 2003). The resulting acetylation of histone tails changes the overall charge of the nucleosome, which likely decreases the inter-nucleosome interaction and the interaction of nucleosome tails with linker DNA, thereby allowing greater chromatin flexibility and thereby accessibility for transcription factors. Moreover, the acetylated lysines work as histone marks, which are recognized by other proteins. Binding of those proteins, typically carrying a bromodomain, to acetylated histones facilitate downstream events resulting in altered gene expression (Hassan et al., 2002; Matangkasombut and Buratowski, 2003).

Histone methylation

Unlike histone acetylation, histone methylation does not alter the overall charge of the nucleosome. To date, there are five lysines

(K) and 3 arginines (R) within histone H3, and one lysine and one arginine within histone H4 that become methylated in a transcription-related manner. An arginine residue can be mono- or di-methylated, whereas a lysine residue can be mono-, di- or tri-methylated. The corresponding histone methyl-transferases display remarkable specificity regarding the level of methylation they catalyze (Sims et al., 2003).

The first identified and best-characterized histone methyl-transferase (HMT) selectively methylates H3-K9 (Rea et al., 2000). This modification correlates with or results in silencing and heterochromatin formation (Heard et al., 2001; Peters et al., 2002). Recent reports suggest that methylation of lysines H3-K27 and H4-K20 also correlate with repressed chromatin structures (Nishioka et al., 2002). In contrast, transcriptionally competent euchromatin is typically methylated at one or more of the three positions, H3-K4, H3-K36 and H3-K79. Di-methyaltion of H3-K4 appears to be a global epigenetic mark in euchromatic regions and tri-methylation of H3-K4 is found in promoters of transcriptionally active genes (Ng et al., 2003c; Santos-Rosa et al., 2002). Methylation of lysines K4, K36 and K79 is also linked to transcript elongation, as will be discussed later in this chapter (Krogan et al., 2003a; Krogan et al., 2003b; Li et al., 2002). Finally, histone H3 and H4 arginine (R) methylation has also been linked to transcriptional activation (Koh et al., 2001; Wang et al., 2001).

As in the case of histone acetylation, histone H3 K9 methylation serves as a "mark" or binding sites for chromatin-associated proteins typically related to heterochromatin protein 1 (HP1) (Bannister et al., 2001; Lachner et al., 2001; Nakayama et al., 2001). These factors

contain chromo-domain modules, which bind to histone tails bearing methyl-lysine in a highly specific manner, with affinity being highest for tri-methyllysine and lowest for mono-methyllysine. Binding of HP1 has been linked to chromatin condensation and gene silencing (Bannister et al., 2001; Lachner et al., 2001; Nakayama et al., 2001).

One very interesting issue, recently also linked to Elongator, which was proposed to have de-methylase activity (Chinenov, 2002), is the mechanism which underlies the removal of methyl-groups. So far, attempts to identify an enzyme with de-methylase activity have not been successful. Early studies concluded that histone methylation is not reversible (Byvoet et al., 1972; Duerre and Lee, 1974). However, this is contradictory to several recent cases, which support the idea that active reversal of methylation may be expected to be necessary to regulate gene expression (Bauer et al., 2002; Nielsen et al., 2001; Santos-Rosa et al., 2002; Strahl et al., 2001).

Histone ubiquitination

In addition to the well-known modifications, such as acetylation and methylation, histones can also be modified through ubiquitination. Histones H2A, H2B, H3 and H1 have been shown to be modified with ubiquitin groups (Chen et al., 1998; Nickel and Davie, 1989; Pham and Sauer, 2000; West and Bonner, 1980). However, only ubiquitination of histone H2B and histone H1 has been so far linked to transcriptional regulation (Kao et al., 2004; Pham and Sauer, 2000). In particular, the co-activator TAFII250 catalyzes the ubiquitination of histone H1 *in vitro* and *in vivo*. This modification is critical for the expression of developmentally regulated genes in *Drosophila* (Pham and Sauer, 2000). Ubiquitination of histone H2B by the Rad6 protein has recently been the focus of attention of numerous studies in yeast (Briggs et

al., 2001; Dover et al., 2002; Krogan et al., 2003a; Ng et al., 2003a; Ng et al., 2003b; Ng et al., 2002b; Sun and Allis, 2002). Moreover, the Ubp8 component of the SAGA complex was identified as the enzyme which catalyses the reciprocal reaction, that is, the removal of ubiquitin from histones (Henry et al., 2003; Kao et al., 2004). All of the above studies support the idea that ubiquitination of histone H2B is linked to transcriptional activation through its impact on other histone modifications.

Histone phosphorylation

Phosphorylation of serine 10 of histone H3 has been linked to transcriptional activation (Cheung et al., 2000b; Lo et al., 2000). Two models have been proposed to explain the effect of histone H3 phosphorylation on gene expression (Cheung et al., 2000a). One possibility is that the addition of negatively charged phosphate groups to the N-terminal H3 tails may disrupt electrostatic interactions between the basic H3 tails and the negatively charged DNA backbone, and thereby increase the accessibility of the underlying DNA to nuclear factors. Another model favors the idea that H3 phosphorylation serves as a recognition "mark" for recruitment of transcription factors or regulatory complexes. Interestingly, proteins participating in receptor tyrosine kinase-mediated signal transduction pathways, bearing an SH2 domain, have been shown to bind phospho-tyrosines (Hunter, 2000). However, to date, no factors that have specific phospho-serine binding motifs have been identified.

The histone code

It is clear from the previous paragraphs that histone tails serve as substrates for extensive posttranslational modifications. These modifications are not just a means of reorganizing nucleosome

structure, but also provide a rich source of epigenetic information. It has been suggested that combinations of specific tail modifications constitute a "code" that defines actual or potential transcriptional states. The code is thought to be set by histone modifying enzymes of defined specificity, and read by non-histone proteins that bind histones in a modification-specific manner (Jenuwein and Allis, 2001; Turner, 2002).

In particular, phosphorylation of serine 10 and acetylation of K9 of histone H3 were found to both correlate with transcriptional activation, and at the same time prevent K9 methylation of histone H3 by Su(var) 3-9, *in vitro* (Cheung et al., 2000b; Lo et al., 2000; Rea et al., 2000). Reduction of K9 methylation was also caused by acetylation of H3 K14. Additionally, functional effects of methylated lysine K20 of histone H4 may be modulated by acetylation of H4 K16 and *visa versa*. It is possible that H4meK20 is a mark associated with chromatin condensation and reduced transcription, whereas H4AcK16 specifies the opposite chromatin state (Rice et al., 2002).

The above examples reveal an interplay of modifications in *cis*. These results were not unexpected, since posttranslational modifications within or adjacent to sites of protein-protein interaction are likely to have some effect on that interaction. However, recent experiments in yeast have revealed a much more surprising relationship. In budding yeast, histone H3 K4 methylation is abolished by mutations in Rad6 or H2B K123 (Dover et al., 2002; Sun and Allis, 2002). As mentioned previously, Rad6 is the major activity responsible for the attachment of ubiquitin to lysine 123 of H2B in yeast. Moreover, *rad6Δ* deletion mutants lose methylation at H3 lysine 4 and 79, modifications found in transcriptional active genes

(Krogan et al., 2003a; Santos-Rosa et al., 2002). This was thus an example of unexpected *trans* interplay between modifications in different histone proteins.

Both *cis* and *trans* interplay of histone modifications have roles in the long-term maintenance and heritability of transcriptional states. However, these modifications have also been associated with more short-term aspects of transcriptional regulation, particularly initiation. Recent data strongly suggest that sequential modifications at defined sites on histone tails facilitate recruitment/binding of transcription-related factors in the assembly pathway (Agalioti et al., 2000; Cosma, 2002; Cosma et al., 1999; Shang et al., 2000).

ATP-dependent chromatin remodeling

ATP-dependent chromatin remodeling is involved in all major reactions performed on chromatin substrates. Its role in activation and repression of transcription affects control of the cell cycle, cell differentiation and hence the development of multi-cellular organisms. Most of the chromatin remodelers are multi-subunit complexes with an ATPase as the catalytic centre. These ATPase subunits can be classified into three families; the SWI2/SNF2-, the ISWI- and the Mi-2/CHD-ATPase families (Varga-Weisz, 2001). They all contain a highly conserved core domain and N- and C- terminal domains, which differ considerably between the above families.

Over the past few years, several models for nucleosome mobility and remodeling have been proposed. The three more prevalent are DNA twisting, which is displayed by members of both SWI/SNF and ISWI classes (Flaus and Owen-Hughes, 2001; Havas et al., 2000; Varga-Weisz and Becker, 1998), structural changes in the

octamer that alters the histone-DNA interactions (Kingston and Narlikar, 1999; Narlikar et al., 2001) and DNA translocation (Saha et al., 2002).

Genes encoding components of the SWI/SNF complex were originally discovered and characterized as pleiotropic transcriptional activators, which fits nicely with the concept that they remodel chromatin in preparation for promoter activation (Abrams et al., 1986; Happel et al., 1991; Hirschhorn et al., 1992). However, more systematic genome-wide analysis showed that only a small fraction of yeast genes require SWI/SNF for activation, and that several genes are also up-regulated in the absence of Swi/Snf, suggesting a role for the complex in both repression and activation (Holstege et al., 1998; Sudarsanam et al., 2000). The finding that only a fairly small number of genes require SWI/SNF for activation contrasts with the notion that the process of transcriptional activation has to contend with a repressive chromatin structure in most if not all genes (Hirschhorn et al., 1992). A possible explanation for this discrepancy was provided by Krebs et al., who proposed that SWI/SNF is globally required for genes expressed in the wake of mitosis, when chromatin is not fully de-condensed (Krebs et al., 2000).

The ATPase ISWI (imitation switch) was first discovered in *D. melanogaster*. Visualization of the protein on polytene chromosomes showed that the bulk of ISWI does not co-localize with the bulk of RNA Polymerase II, which indicated that transcriptional regulation is unlikely to be the main role of ISWI, at least in the specialized salivary gland tissue (Deuring et al., 2000). However, in *in vitro* model reactions, ISWI-containing remodeling factors can stimulate transcription from chromatin templates (Ito et al., 1997). Three

ISWI-containing remodeling complexes have been discovered in *D. melanogaster* following different biochemical assays; NURF (nucleosome remodeling factor), ACF (ATP-utilizing chromatin assembly and remodeling factor) and CHRAC (chromatin accessibility complex) (Eberharter et al., 2001; Varga-Weisz et al., 1997; Xiao et al., 2001). All three complexes are able to induce the sliding of nucleosomes to neighbouring DNA segments, thereby rendering DNA segments accessible to interacting factors, and facilitating transcription initiation.

Like ISWI, the CHD ATPase is able to disrupt histone-DNA interactions and to induce nucleosome sliding on DNA fragments (Brehm et al., 2000; Wang and Zhang, 2001). The more intensively studied CHD3 (Mi-2α) and CHD4 (Mi-2β) contain two PHD fingers-presumed protein recognition modules in addition to the chromo-domains. Mi-2 was first identified as a dermatomyositis-specific antigen and later shown to reside in so-called NRD/NuRD/NURD complexes in various species (Knoepfler and Eisenman, 1999). The name of this complex (Nucleosome Remodeling and De-acetylation) hints at the subunit composition: in addition to Mi-2 and several other subunits, a module consisting of the histone de-acetylases HDAC1/2 and the H4 interacting proteins RbAp46/48 are also present (Guschin et al., 2000). NURD complexes are of considerable interest since they physically combine aspects of the two fundamental strategies for chromatin remodeling: covalent modification (de-acetylation) and ATP-dependent remodeling. This combination suggests that energy might also be spent to render the histone N-termini accessible for coordinated de-acetylation. Consistent with this idea, the ATPase activity of Mi-2 can increase the efficiency of histone de-acetylation

by NURD complexes in cell-free systems (Guschin et al., 2000; Tong et al., 1998). Since hypo-acetylated histone N-termini are generally correlated with repression of transcription, NURD complexes were suspected of being involved in establishing a repressive chromatin environment. This idea was also supported by identification of a mammalian NURD subunit, the MBD3 protein (Wade et al., 1999; Zhang et al., 1999). This protein is highly related to the methyl-cytosine binding protein MBD2. MBD2 itself can associate with NURD to form the previously known MeCP1 complex, which preferentially interacts with DNA methylated at CpG residues (Ng et al., 1999). Through interaction with MBD2, NURD is targeted to bind, remodel and de-acetylate nucleosomes containing methylated DNA, providing a possible mechanism by which DNA methylation may lead to silencing of genes.

1.1.5 The mechanism of RNA Polymerase II transcript elongation

Though the transcription initiation step has almost monopolized the focus of studies over the past 20 years, interest in transcript elongation and termination has recently grown. It has thus become obvious that all the three basic steps of transcription are closely linked to ensure the accurate synthesis of mRNAs. The main focus of this section is to present the current view about the regulation of transcript elongation and the proteins, which are thought to play crucial roles in this process.

TFIIS

The first RNA Polymerase II elongation factor to be purified was named TFIIS (transcription factor II S) and was initially

identified by its ability to promote efficient synthesis of long transcripts *in vitro* (Sekimizu et al., 1976). Biochemical studies subsequently revealed that TFIIS promotes elongation by preventing premature arrest of polymerase at discrete sites within transcribed DNA sequences (Reines et al., 1989). The proposed mechanism for that process favours a direct interaction of SII with RNAPII and the transcript itself. TFIIS reactivates arrested RNA Polymerase II by triggering RNAPII-mediated cleavage of the incorrectly positioned nascent RNA in the polymerase active site, a process which realigns the active site with the end of the RNA and allows release of the polymerase from the site of arrest (Izban and Luse, 1992; Rudd et al., 1994; Wang and Hawley, 1993). Consistent with the above model, recent structural data reveal that SII extends from the polymerase surface *via* a pore to the internal active site and interacts with RNA Polymerase II at sites near the pore (through which nucleotides are thought to enter the RNA Polymerase II active site) (Cramer et al., 2000; Cramer et al., 2001; Gnatt et al., 2001; Kettenberger et al., 2003). Moreover, SII was found to be cross-linked to the 3'-end of transcripts in the arrested RNA Polymerase II elongation complex (Powell et al., 1996).

Yeast cells lacking the *SII* (*DST1/PPR2*) gene exhibit transcriptional defects when grown in the presence of the drug 6-azauracil, an inhibitor of GTP and UTP metabolism. These defects are synergistically enhanced when the *SII* deletion was combined with mutations in genes encoding RNA Polymerase II subunits or other transcription elongation-specific factors, such as DSIF/NELF, Elongator and FACT (Hemming et al., 2000; Lennon et al., 1998; Lindstrom and Hartzog, 2001). More recently, chromatin

immunoprecipitation experiments from yeast confirmed that SII is present along the promoter and coding regions of multiple genes (Pokholok et al., 2002). Finally, SII has been shown to affect yeast transcription *in vivo*, by stimulating the passage of RNA Polymerase II through an artificial arrest site (Kulish and Struhl, 2001).

TFIIF, ELL, ELONGIN

Efforts to identify activities that stimulate the rate of elongation by RNA Polymerase II on naked DNA templates, led to the purification of TFIIF and members of the ELL and Elongin families. Their mode of action includes suppression of transient pausing of RNAPII at all or most steps of nucleotide addition (Shilatifard, 1998; Shilatifard et al., 1996). *In vitro* studies have suggested that this is achieved by helping to maintain the 3'-OH terminus of the nascent transcript in proper alignment with the catalytic site of polymerase, thereby preventing backtracking by the enzyme (Elmendorf et al., 2001; Gu and Reines, 1995; Takagi et al., 1995; Wang and Hawley, 1993). Consistent with this idea, TFIIF, ELL and Elongin complexes have been shown to inhibit SII-induced cleavage of nascent transcripts by paused RNAPII (Elmendorf et al., 2001).

Studies in cells, however, have suggested that these three factors (also) serve quite different functions. Apart from a role in pre-initiation complex formation (PIC), which was mentioned previously, more recent work revealed a function for TFIIF in promoter escape as well (Yan et al., 1999). TFIIF was found to play a major role in reducing the frequency at which RNAPII aborts transcription during synthesis of the first few phosphodiester bonds of nascent transcripts, ensuring that they reach a sufficient length

(>6 to 8 nucleotides) to be relatively resistant to abortion. Consistent with this role, chromatin immunoprecipitation experiments in *S. cerevisiae* cells revealed that TFIIF localizes only near promoter regions and dissociates from RNA Polymerase II elongation complexes shortly after transcription initiates (Pokholok et al., 2002). Therefore, by ensuring an efficient promoter escape, TFIIF might stimulate the rate of transcription.

In contrast, the founding member of the ELL family, Ell, seems to have a more direct role in transcript elongation. For example, *Drosophila* Ell was found at a large number of transcriptionally active sites on polytene chromosomes and was rapidly re-localized with RNA Polymerase II to heat shock genes when embryos were subjected to elevated temperatures (Gerber et al., 2001). Moreover, as expected for a *bona fide* elongation factor, *Ell* mutations in *Drosophila* preferentially affect expression of large genes, such as the *Cut*, the *Notch* and the *Sex combs reduced* genes, but not shorter genes (Eissenberg et al., 2002).

Finally, Elongin is a heterotrimeric protein complex composed of a transcriptionally active A subunit and two smaller subunits, Elongin B and C, which can form a discrete BC sub-complex (Aso et al., 1995; Garrett et al., 1994). The A subunit has been shown to bind to the Elongin BC complex through a sequence motif referred to as the BC-box (Aso et al., 1995; Garrett et al., 1994). Recent evidence has identified a novel role for Elongin. Elongin A was shown to be linked *via* Elongin B and C to a heterodimeric module composed of Cullin family proteins and the RING-H2 finger protein Rbx1. These multi-subunit complexes have a function as E3 ubiquitin ligase, opening the possibility that Elongin might ubiquitylate proteins during

transcription (Kamura et al., 2001). Consistent with a role for Elongin subunits in transcription-coupled protein ubiquitylation, the Mediator subunit Med8 interacts with Elongin BC and assembles into an ubiquitin ligase in mammalian cells (Brower et al., 2002).

Elongation factors that target the C-terminal domain of Rpb1

The transition from transcription initiation to elongation is accompanied by changes in the phosphorylation status of the C-terminal domain (CTD) of the largest subunit (Rpb1) of RNAPII. In fact, the transition correlates perfectly with the hyper-phosphorylation of the CTD (Dahmus, 1996). The current model proposes that the form of RNA Polymerase II, which enters promoter is un- (or at least hypo-) phosphorylated ($PolII_A$), whereas after the initiation/elongation transition (promoter clearance) the polymerase is hyper-phosphorylated ($PolII_O$). The predominant sites of phosphorylation are at serine 2 and serine 5 of the CTD repeat. Chromatin immunoprecipitation experiments, using antibodies that specifically recognize either of these two sites showed that phosphorylated serine 5 is predominantly found on polymerases close to the promoter (promoter escape/early elongation). However, as elongation continues, the phosphoserine 5 signal seems to slowly be lost while the serine 2 position becomes highly phosphorylated (Komarnitsky et al., 2000). It is believed that the CTD serves as a scaffold for factors involved in different stages of transcription and that the phosphorylation transitions regulate the shift from initiation- to elongation-specific factors that are associated with polymerase.

Initial efforts to understand the role of CTD phosphorylation in transcription were aided greatly by protein kinase inhibitors, such as the 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB), which can block CTD phosphorylation and induce arrest of elongating RNA Polymerase II *in vivo* and in crude transcription systems *in vitro* (Chodosh et al., 1989; Marshall and Price, 1992). These studies predicted the existence of two classes of elongation factors responsible for DRB-sensitive transcription: a class of negatively acting factors that inhibit transcription and a class of positively acting factors that overcome this inhibition. Fractionation of crude DRB-sensitive transcription systems led to the discovery of three novel elongation factors: the P-TEFb (Positive Transcription Elongation Factor b) (Marshall and Price, 1995), which is a positively acting, DRB-sensitive factor and two negatively acting factors, NELF (Negative Elongation Factor) (Yamaguchi et al., 1999) and DSIF (DRB Sensitivity Inducing Factor) (Wada et al., 1998).

P-TEFb is composed of the ~43kD Cdk9 kinase (also referred to as PITALRE) and one of several cyclins (T1, T2a, T2b or K). This kinase is capable of phosphorylating the CTD of RNA Polymerase II (Marshall et al., 1996) and the Spt5 subunit of DSIF (Kim and Sharp, 2001). The first evidence implicating P-TEFb kinase in regulation of transcription elongation *in vivo* came from the finding that P-TEFb is recruited to early RNA Polymerase II elongation complexes, which were engaged in synthesis of the human immunodeficiency virus-1 (HIV-1) transcripts. This recruitment is mediated *via* the interaction of cyclin T subunit with the HIV-1 encoded Tat protein (Wei et al., 1998). Later on, P-TEFb was found to be broadly distributed at sites of transcription on *Drosophila* polytene chromosomes and rapidly

redistributed to heat shock genes when they were activated by temperature shift (Lis et al., 2000). Moreover, RNAi experiments in *C. elegans* showed abolishment of CTD serine 2 phosphorylation by inactivating P-TEFb, strongly suggesting that a major substrate for this kinase is serine 2 of the CTD (Shim et al., 2002).

Yeast proteins with functional similarity to Cdk9 are the Bur1 and Ctk1 kinases. The latter phosphorylates serine 2 of the CTD (Cho et al., 2001). The former has been shown to prefer serine 5 *in vitro* (Keogh et al., 2003). However, mutations in *BUR1* do not significantly affect the phosphorylation state of either serine 2 or serine 5, indicating an *in vivo* substrate for Bur1 other than RNAPII. Consistent with a role in elongation, both Ctk1 and Bur1 (as well as its cyclin partner Bur2) are associated with transcribed chromatin (Keogh et al., 2003).

The two negatively acting factors, DSIF and NELF, were purified from HeLa cells. The former was found to be composed of two subunits (Spt4 and Spt5), ubiquitously expressed in eukaryotes from yeast to man (Wada et al., 1998), and the latter of five subunits, which are all required for inhibition of RNA Polymerase II elongation (Yamaguchi et al., 1999). Although the precise mechanism of action for both factors is unknown, it likely involves a direct interaction with polymerase and the nascent transcript (Yamaguchi et al., 2002). More recently, it was suggested that DSIF and NELF associate with the promoter of *hsp70* in *Drosophila* before heat shock, and cause un-phosphorylated RNA polymerase II (IIA) to pause in promoter proximal regions. Upon elevation of the temperature, the HSF activator contributes to dissociation of NELF from the elongation

complex, whereas DSIF remains associated and could serve a positive role in elongation (Wu et al., 2003).

In all likelihood, efficient elongation requires that the CTD is kept phosphorylated during the entire length of the run. However, as soon as one cycle of transcription is finished, RNAPII has to be recycled for utilization in a new round of transcription. This requires de-phosphorylation of the CTD. This process is not yet well defined. The best-characterized CTD phopshatase so far is the Fcp1 protein. Fcp1 was shown to associate with elongating RNAPII *in vivo* at both promoters and coding regions of genes (Cho et al., 2001). The same study showed, that in yeast, Ser2 phosphorylation increases, while Ser5 phosphorylation levels are relatively unchanged in the absence of functional Fcp1. In contrast, human Fcp1 has been shown to be capable of de-phosphorylating both serine 2 and serine 5 residues of the C-terminal domain of Rpb1 *in vitro* (Lin et al., 2002). Finally, it should be mentioned that two novel CTD phosphatases have recently been identified. Both these enzymes, Scp1 and Ssu72, show a preference for phospohrylated serine 5 residues (Krishnamurthy et al., 2004; Yeo et al., 2003).

Factors which facilitate transcript elongation through chromatin

The need for RNA Polymerase II to transcribe through chromatin *in vivo* led investigators to identify factors that facilitate this process. The most prominent of those are the Spt family of proteins, the FACT complex, the Swi/Snf family of proteins and the Elongator complex (which will be discussed in detail later).

SPT genes were first identified in *S. cerevisiae* by screening for genetic suppressors of certain promoter insertion mutations

(Fassler and Winston, 1988; Simchen et al., 1984). One class of *SPT*, the histone class, includes *SPT4, SPT5, SPT6, SPT16, SPT11* and *SPT12*. The last two encode the histones H2A and H2B. The genes of this class share a number of mutant phenotypes that inspired the view that these proteins express their function through effects on chromatin (Malone et al., 1991; Swanson and Winston, 1992). When mutations in *SPT4, SPT5* or *SPT6* genes are combined with a deletion of the *DST1/PPR2* gene (which encodes the transcription factor TFIIS), the cells display conditional growth defects. Moreover, deletion of *SPT* genes renders cells sensitive to the drug 6-azauracil, a transcript elongation-specific inhibitor (Hartzog et al., 1998). Consistent with a role in transcript elongation, several transcripts show decreased levels in the *spt5Δ* cold-sensitive mutants. Interestingly, the Spt4, Spt5 and Spt6 proteins co-immunoprecipitate with each other and with RNA Polymerase II (Hartzog et al., 1998), co-localize on *Drosophila* polytene chromosomes in a pattern that closely resembles that observed for PolIIO (hyper-phosphorylated form, engaged in elongation), and are recruited to the promoter and transcribed regions of heat shock genes upon elevation of temperature (Kaplan et al., 2000). Recently, Kaplan et al. demonstrated an *in vivo* requirement for Spt6 for maintaining normal chromatin structure during transcript elongation, thereby repressing transcript initiation from cryptic sites (Kaplan et al., 2003).

Using recombinant or highly purified transcription factors, DNA assembled into chromatin and HeLa nuclear extracts, Orphanides et al. established an *in vitro* system for transcript elongation through chromatin (Orphanides et al., 1998). Using this system, a complex of proteins named FACT (Facilitates Chromatin Transcription) was

purified based on its ability to support efficient elongation through chromatin-bound DNA. FACT is composed of two subunits, hSPT16 and SSRP1, which are homologues of yeast proteins Cdc68/Spt16 and Pob3, respectively (Orphanides et al., 1999). FACT functions optimally when present in near stoichiometric quantities to nucleosomes, by promoting the disassembly of one H2A/H2B dimer from the nucleosome, thus de-stabilizing its structure to allow RNAPII passage (Orphanides et al., 1999). FACT was also found to have histone chaperone activity, suggesting that it plays a role in re-establishing chromatin structure after RNAPII traverses a nucleosome (Saunders et al., 2003). Finally, FACT interacts genetically and biochemically with other transcript elongation-related factors, such as Spt4, Spt5 and the Paf1 complex (Costa and Arndt, 2000; Formosa et al., 2002; Krogan et al., 2002b; Squazzo et al., 2002).

The role of ATP-dependent chromatin-remodeling complexes during transcript initiation has been studied extensively. However, in the last few years a number of results have revealed an important and unique role for these complexes during transcript elongation as well. Studies in mammalian systems suggest such a role for the Swi/Snf complex. In particular, using as a model the *hsp70* from mammalian cells in an *in vitro* system, Brown et al. identified mutations in the activation domain of the activator HSF1, which were required to release paused RNA polymerase II on a nucleosomal template (Brown et al., 1996). These mutations abolished the interaction of HSF1 with the Swi/Snf complex, indicating a role for the latter in the remodeling of chromatin adjacent to polymerase pause sites on the *hsp70*. More recently, work with immortalized fibroblasts showed that the previously mentioned mutations in HSF1 activator are capable of

directing RNAPII transcription initiation on the *hsp70* promoter to the same degree as wild-type HSF1, but that the mutants cannot support Swi/Snf recruitment, remodeling or subsequent *hsp70* induction (Corey et al., 2003). Thus, localized recruitment of Swi/Snf to the *hsp70* by an activator occurs concurrently with localized chromatin remodeling and stimulation of elongation (Corey et al., 2003). Finally, in yeast, deletion mutants for genes encoding Swi/Snf subunits were found to be synthetically lethal when combined with a mutation in the *DST1/PPR2*, which encodes the transcription elongation factor TFIIS, further suggesting a role for components of the Swi/Snf complex in transcript elongation (Davie and Kane, 2000).

It should be mentioned that the transcription-coupling repair factor CSB (Cockayne's syndrome B) and its yeast homologue Rad26 also belong to the Swi/Snf family of proteins. CSB enhances transcript elongation on naked DNA *in vitro* (Selby and Sancar, 1997), and chromatin remodeling *in vivo* (Citterio et al., 2000). Interestingly, Rad26, the yeast homologue, was also suggested to promote RNA Polymerase II transcript elongation *in vivo* (Lee et al., 2001; Lee et al., 2002).

Other recently identified proteins with proposed roles in transcript elongation

Proteomic approaches have been used to identify a large network of protein-protein interactions between RNAPII and transcript elongation factors. In most cases, these observations were complemented by strong genetic interactions that are indicative of shared functions and of potential roles in transcript elongation.

The Paf1 complex consists of Paf1, Cdc73, Hpr1, Ccr4, Ctr9, Rtf1 and Leo1 and was originally isolated as an RNAPII-associated factor (Krogan et al., 2002b). The genes that encode the Paf1 complex components are not essential. However, mutations in these genes resulted in changes in abundance of a small but significant subset of yeast transcripts (Krogan et al., 2002b). Moreover, genetic and biochemical data supported the idea that the Paf1 complex travels along the coding region of transcribed genes and mediates interactions of RNAPII with chromatin (Krogan et al., 2002b; Squazzo et al., 2002).

The Chd1 protein is an ATP-dependent chromatin remodeling enzyme, which has been shown by affinity purification, co-immunoprecipitation and two-hybrid experiments to interact with the Paf1 and the FACT complex (Kelley et al., 1999; Krogan et al., 2002b; Mueller and Jaehning, 2002; Tran et al., 2000). Localization studies in *Drosophila* showed that Chd1 associates with transcriptionally active genes *in vivo* (Stokes et al., 1996), whereas the yeast homolog of Chd1 was found to be recruited to coding regions of transcribed genes (Krogan et al., 2002b).

Another ATP-dependent chromatin remodeling enzyme, Isw1, was also recently shown to have a role in transcript elongation (Morillon et al., 2003a). Isw1 is an ATPase, which is found in two distinct complexes, Isw1a and Isw1b. The Isw1a complex was shown to repress gene transcription at initiation through specific positioning of a promoter proximal di-nucleosome, whereas the Isw1b complex acts within coding regions to control the amount of RNA Polymerase II released into productive elongation and to coordinate elongation with termination and pre-mRNA processing. Interestingly, these effects

are controlled *via* phosphorylation of the CTD of RNAPII and methylation of the chromatin template (Morillon et al., 2003a).

Finally, members of the winged-helix forkhead (Fkh) family of proteins were recently reported to be involved in regulation of transcript elongation (Morillon et al., 2003b). In yeast, there are two genes, which encode Fkh factors, *FKH1* and *FKH2*. Both proteins were found to be associated with coding regions of active genes, and influence, in opposing ways, transcript elongation and termination. It was proposed that the opposing action of Fkh1 and Fkh2 may be part of a checkpoint mechanism to coordinate transcription and pre-mRNA processing *via* CTD phosphorylation (Morillon et al., 2003b).

Transcript elongation and chromatin modification

As discussed previously, histone modifications have been linked very strongly to regulation of gene expression. In the last few years, a number of studies have proposed a link between the methylation status of lysines 4, 36 and 79 of histone H3 and transcript elongation. In yeast, methylation of lysine 4 of histone H3 is catalyzed by the histone methyl-transferase (HMT) complex named COMPASS (COMplex of Proteins Associated with Set1) (Briggs et al., 2001; Krogan et al., 2002a; Miller et al., 2001). COMPASS was found to interact with the Paf1 complex and the form of RNA Polymerase II phosphorylated at serine 5 of the CTD. Both the interaction with RNA Polymerase II and the methylation of lysine 4 require the presence of the Paf1 complex (Krogan et al., 2003a; Ng et al., 2003b). Methylation of lysine 4 of histone H3 and members of the COMPASS complex have been shown to localize to the 5' regions of active mRNA coding genes. Their recruitment is dependent on TFIIH-associated kinase (Kin28),

which is the kinase that phosphorylates the RNAPII CTD at serine 5 and mediates the transition from initiation to elongation (Ng et al., 2003b).

Methylation of lysine 36 is catalyzed by another SET domain-containing protein in yeast, Set2 (Strahl et al., 2002). In an effort to understand the role of Set2, different groups have shown that Set2 interacts directly with RNA Polymerase II, preferentially with the form phosphorylated at serine 2 of the CTD (Krogan et al., 2003b; Li et al., 2003; Li et al., 2002; Xiao et al., 2003). This interaction requires the presence of the CTD, the Paf1 complex and the CTD serine 2 kinase, Ctk1. Moreover, Ctk1 kinase is required for efficient methylation of lysine 36, revealing a possible targeting of Set2 to coding regions of transcribed genes *via* the CTD phosphorylation activity of this kinase.

Finally, methylation of lysine 79 of yeast histone H3 is catalyzed by a protein named Dot1 (Lacoste et al., 2002; Ng et al., 2002a; van Leeuwen et al., 2002). Although this modification was first hypothesized to exclusively regulate the repression of genes in telomeric regions, recent reports show that methylation of lysine 79 occurs in euchromatic regions as well. As for lysine 4 methylation, ubiquitination of lysine 123 of histone H2B is also required for methylation of lysine 79 of histone H3 (Ng et al., 2002b). Consistent with a role for the Paf1 complex as a "platform" for interaction of histone methyl-transferases with elongating RNA Polymerase II, methylation of lysine 79 is also dependent on the presence of components of the Paf1 complex (Krogan et al., 2003a). However, so far only genetic interactions between components of the Paf1 complex and Dot1 have been observed. The presence of Paf1 at both promoters

and coding regions of actively transcribed genes suggested a possible role for methylation at lysine 79 of histone H3 in both early and late transcript elongation (Krogan et al., 2003a).

The identification of methylated histone residues allowed the investigators to propose a model for a role of histone methylation as a mark of "transcriptional memory" for recently transcribed genes. According to this model, histone methylation at the promoter and the coding region of genes inform the cell that transcription of a given gene has occurred and how far RNA Polymerase II has transcribed through the body of that gene in the recent past (Gerber and Shilatifard, 2003).

1.1.6 Coordinating roles for RNAPII elongation complex in mRNA processing and export

A number of recent studies have supported the notion that RNA Polymerase II is not simply an RNA synthesis machine, but that it also serves a more fundamental role as a platform for recruiting and coordinating the actions of proteins responsible for mRNA maturation and export to the cytoplasm.

Both hyper- and hypo-phosphorylated forms of CTD stimulate 3' cleavage *in vitro* (Hirose and Manley, 1998; Ryan et al., 2002), whereas only the hyper-phosphorylated form stimulates pre-mRNA splicing and capping *in vitro* (Hirose and Manley, 1998; Hirose and Manley, 2000). Moreover, protein interaction studies have shown that splicing factors as well as components of the capping machinery interact directly with the phosphorylated form of RNA Polymerase II (Ho and Shuman, 1999; Mortillaro et al., 1996). In addition, results of chromatin immunoprecipitation experiments indicate that components of the

mRNA capping complex are transiently associated with the promoter and the coding region of transcribed genes (Takase et al., 2000). Interestingly, the Ssu72 protein was found to be an integral component of the CPF polyadenylation complex (Dichtl et al., 2002). *ssu72Δ* mutant cells display defects in RNAPII transcript elongation and termination, thereby linking the two processes. Finally, Ssu72 was also shown to be a CTD phosphatase, reinforcing the idea that the CTD and its modifications are instrumental in ensuring correct mRNA maturation (Krishnamurthy et al., 2004).

In order to be translated, the newly synthesized mRNA has to be transported to the cytoplasm. A link between the RNAPII elongation complex and the mRNA export machinery was recently brought to light with the identification of the yeast THO/TREX complex (Chavez et al., 2000). This complex was shown to interact with free and transcribing RNA polymerase II and cells carrying mutations in its subunits exhibit apparent defects in RNA Polymerase II transcript elongation of both long and G+C rich transcripts, and in nucleocytoplasmic transport of properly processed mRNAs (Chang et al., 1999; Chavez et al., 2001; Jimeno et al., 2002; Strasser et al., 2002). These recent findings raised the intriguing possibility that the THO complex may recruit the mRNA export machinery directly to transcribing RNAPII to facilitate the transport of mRNA from the nucleus to their sites of translation in the cytoplasm.

As should be obvious from the above overview on the transcription cycle, the last few years have seen the emergence of very convincing data to suggest that cells have put in place sophisticated checkpoint mechanisms to ensure that transcription is productive. In other words, all the steps from initiation of

transcription to RNA export are extraordinarily inter-linked, so that when a transcription cycle starts this will lead to translatable mRNA. This model is sensible, but at the same time very complicated, since it predicts inter-communication between different protein complexes. Several labs are working on this extremely interesting model, trying to reveal the functional network that cells utilize to achieve co-ordinated regulation of gene expression.

1.2 ***Elongator complex***

The Elongator complex, which is the focus of this thesis, has been isolated from both *S. cerevisiae* and human cells. The exact role of this complex remains unclear, but several lines of evidence indicate a role in regulation of chromatin modification, and as a co-factor for RNA Polymerase II during elongation. Below is presented an overview of the Elongator literature. This overview will also be useful as an introduction for the next Chapters and the work that is described in this thesis.

Purification of yeast Elongator complex

As mentioned previously, the form of RNA Polymerase II engaged in transcript elongation is hyper-phosphorylated on the CTD of the largest polymerase subunit, Rpb1. The ternary RNAPII/DNA/RNA complex is extremely stable and, thus, associates with chromatin in a salt-stable manner (Svejstrup et al., 1997). In an attempt to identify new transcription elongation-specific factors, Otero et al. took advantage of these features to isolate the hyper-phosphorylated, elongating form of RNAPII from yeast chromatin (Otero et al., 1999). Three polypeptides with apparent molecular

weights of 150, 90 and 60 kDa were found to co-purify with the polymerase. Mass spectrometry analysis revealed the identity of these proteins and the genes that encode them, which were named *ELP1* (for Elongator protein 1), *ELP2* and *ELP3*, respectively (Fellows et al., 2000; Otero et al., 1999; Wittschieben et al., 1999).

When Elp1 protein was purified by conventional means from the soluble fraction of yeast extracts it was found to associate directly with the Elp2 and Elp3 proteins. The three-subunit complex was named "Elongator" and shown to associate directly with the hypo- and hyper-phosphorylated form of RNAPII. However, binding was more stable with hyper-phosphorylated RNAPII (Otero et al., 1999).

To further investigate the role of the Elongator complex in transcript elongation, yeast deletion mutant strains for each of the *ELP* genes were constructed and tested for a number of phenotypes (Fellows et al., 2000; Otero et al., 1999; Wittschieben et al., 1999). These mutant cells displayed only a slightly slower growth rate than wild type under normal conditions (glucose), but showed very prominent delays in their ability to adjust to growth in media containing other carbon sources, such as galactose, sucrose or raffinose. Additionally, all the *elpΔ* mutants were sensitive to high salt concentrations (1 M NaCl) in the growth medium. Northern blot analysis showed that these phenotypes could be directly correlated with transcription defects *in vivo*. In fact, experiments studying the kinetics of transcription of several inducible genes revealed that they displayed a reduced rate of induction, most prominently at the early time points. In contrast, the constitutively expressed genes tested were unaffected (Fellows et al., 2000; Otero et al., 1999; Wittschieben et al., 1999).

The deletion mutants for all of the three initially identified Elongator genes also exhibited other phenotypes, such as temperature sensitivity and sensitivity towards the transcript elongation inhibitors, such as 6-AU (azauracil) and mycophenolic acid. Interestingly, when an *ELP* deletion was combined with a deletion of the gene that encodes the transcript elongation factor TFIIS (*DST1*), the *elpΔ/dst1Δ* double mutant cells displayed hypersensitivity to those inhibitors. Sensitivity to these compounds is a common characteristic of cells lacking transcript elongation-specific factors, such as TFIIS and Spt4/Spt5, suggesting a synergistic role of Elongator and TFIIS in transcript elongation (Fellows et al., 2000; Otero et al., 1999; Wittschieben et al., 1999).

Database searches revealed that the *ELP* genes have well conserved homologues from yeast to man (Fellows et al., 2000; Otero et al., 1999; Wittschieben et al., 1999). Computational analysis of the Elp1 protein identified domains with weak homology to WD40 and Tetratricopeptide repeats (TPR) (Otero et al., 1999). More recently, it has also been proposed that yeast Elp1 has a putative nuclear localization signal (NLS) at the C-terminus (Fichtner et al., 2003). Sequence analysis of yeast Elp2 and Elp3 uncovered eight WD40 repeats for the former, and three C-terminal motifs for the latter that are conserved among acetyl-transferases in the GNAT (Gcn5-related N-Acetyl-Transferase) super-family (Fellows et al., 2000; Wittschieben et al., 1999). Interestingly, Elp3 was also recently found to have a N-terminal domain similar to the catalytic domain of S-adenosylmethionine (SAM) radical enzymes, suggesting that the Elp3 might be involved in de-methylation, for example of histones (Chinenov, 2002). Whether Elongator possesses histone de-

methylation activity is currently under investigation in the Svejstrup laboratory.

The use of *in-gel* HAT assays suggested that recombinant Elp3 was capable of acetylating all four histones at their amino-terminal tails (Wittschieben et al., 1999). Upon mutating two conserved tyrosine residues that are predicted to be directly involved in acetyl-CoA binding, this HAT activity was significantly reduced. In addition, expression of Elp3 carrying either of these mutations could not complement the phenotype of cells lacking *ELP3*. However, the mutated Elp3 molecules could still incorporate into Elongator complexes, suggesting an important role for the acetyl-transferase activity of Elp3 in Elongator function *in vivo* (Wittschieben et al., 2000).

Importantly, cells lacking the *ELP3* gene were inviable in the absence of the histone H4 N-terminal tail, and temperature sensitive when they lacked the histone H3 tail. The interpretation of this result was that Elp3 is mainly involved in H3 and to a lesser extent in H4 acetylation *in vivo* (Wittschieben et al., 2000). Additionally, *elp3Δ* cells were also more temperature sensitive when they carried point mutations in histone H3 tail sequences encoding the "transcription-related" lysine residue 14, or the H4 lysine residues 8 and 16. In contrast, this phenotype was not observed when the *elp3Δ* deletion was combined with mutations in residues primarily thought to be related to nucleosome deposition (H3 K9R; H4 K5, 12R), further suggesting a role for Elp3 in transcription-associated chromatin modification and remodeling (Wittschieben et al., 2000).

Yeast holo-Elongator is a six-subunit complex

As described above, the Elongator complex isolated from the chromatin fraction of yeast whole cell extract was initially thought to be a three subunit complex. However, when affinity tagged Elp1 protein was purified from the soluble fraction, six proteins were found to co-elute in a stoichiometric manner (Winkler et al., 2001). The three new proteins were named Elp4, Elp5 and Elp6. Gel filtration chromatography confirmed that all six proteins exactly co-purify. Experiments, which further support the idea that Elongator is a six-subunit complex, will be presented in Chapter 3.

Database searches revealed a number of *ELP4* homologues in higher eukaryotes, including human and mouse (Winkler et al., 2001). Interestingly, the human and mouse Elp4 proteins are encoded by the human and mouse *PAXNEB* genes. The human gene is localized in a region implicated in the human disease *WAGR* syndrome (Wilm's tumour, Aniridia, Genitourinary abnormalities and mental Retardation). Although simple BLAST database searches with the *ELP5* and *ELP6* genes revealed no apparent similarity to any sequence of other organisms, more advanced searches identified orthologues across eukaryotes, in fungi, *C. elegans*, *Drosophila* and mammals (Ponting, 2002). The precise function of the Elp4, Elp5 and Elp6 proteins remains unclear. However, sequence-based analysis suggested that both Elp4 and Elp6 are distant homologues of P-loop ATPases/GTPases, in which the ATPase/GTPase activity has been inactivated (Ponting, 2002). The work that will be presented in Chapter 3 aims to reveal roles for these three proteins in the context of the Elongator complex.

The purification of a six subunit Elongator complex from the soluble fraction of yeast extracts raised the question of why the complex between RNAPII and Elongator, purified from the chromatin fraction was apparently composed only of three Elongator proteins (Elp1, Elp2 and Elp3). One obvious possibility was that chromatin-bound Elongator is also a six-subunit complex, but that it is disrupted either by anion-exchange chromatography or by the high salt concentrations used during the purification procedure. Indeed, when the six-subunit purified complex from soluble fraction was subjected to Mono Q chromatography and eluted with increasing salt concentrations, it was found to be disrupted into two sub-complexes, a "large" core-Elongator complex composed of Elp1, Elp2 and Elp3 proteins and a "small" sub-complex, composed of Elp4, Elp5 and Elp6 (Winkler et al., 2001). Moreover, as will be shown in Chapter 3, affinity resin-associated holo-Elongator could be disrupted into the two mentioned sub-complexes upon treatment with high salt. Thus, holo-Elongator is a fragile multi-subunit complex, explaining the failure to identify all the six subunits in the RNA Polymerase II holo-enzyme originally isolated from yeast chromatin.

Concomitantly with Winkler et al., two other groups also isolated the six-subunit yeast Elongator complex from the soluble fraction, by following different purification strategies (Krogan and Greenblatt, 2001; Li et al., 2001). Moreover, microarray analysis of gene expression in *elpΔ* strains, performed by Krogan and Greenblatt (2001) showed that, in the *elpΔ* cells a subset of genes had reduced and that another subset of genes had increased expression levels, compared to wild type. More importantly, the effects of the *elp4Δ* and *elp6Δ* mutations on mRNA expression correlated almost perfectly

with the effects of *elp1Δ* and *elp2Δ*, strongly indicating that the two sub-complexes have similar effects on gene expression, and thereby further supporting the idea that all Elp proteins function in the same complex (Krogan and Greenblatt, 2001).

In an attempt to make a crude map of interactions between Elongator subunits, Schaffrath and co-workers performed Elongator immunoprecipitation experiments using extracts from wild type and *elpΔ* mutant cells (Fichtner et al., 2002b; Frohloff et al., 2003). Due to a lack of antibodies against all the Elp proteins, the authors were unable to make firm conclusions about the binary interactions between Elongator subunits. The experiments described in Chapter 3 present the first comprehensive analysis of the molecular architecture of yeast Elongator complex.

Overlapping roles for the histone acetyl-transferase activities of SAGA and Elongator in vivo

The purified yeast holo-Elongator complex was used in *in vitro* HAT assays, which showed that it has a strong preference for the histone H3 tail and to a lesser extent the H4 tail, either in the context of core histones or nucleosomes (Winkler et al., 2001). Edman-sequencing was used to identify the acetylation target sites. The predominant target was found to be lysine 14 of histone H3 and to a lesser extent lysine 8 and lysine 12 of histone H4. More importantly, core-Elongator, composed of only Elp1, Elp2 and Elp3, had no activity, suggesting that the Elp4/Elp5/Elp6 sub-complex is required to make Elongator an active HAT (Winkler et al., 2001).

Combining the *ELP3* deletion with *GCN5* deletion, (*GCN5* encodes a well-characterized transcription-related HAT), gave rise to

severe synthetic growth phenotypes (Wittschieben et al., 2000). In contrast to the single mutants, the *elp3Δ gcn5Δ* double mutant cells were unable to grow on galactose-, raffinose- or sucrose-containing medium. The same was true for growth at high temperature (37°C). Further studies showed that the functional overlap between Gcn5 and Elp3 was due to their intrinsic HAT activity, as even combination of single point mutations in the active sites of these proteins resulted in severe growth defects. Remarkably, mutation of specific de-acetylases (*HOS2 and HDA1*) suppressed the *elp3Δ gcn5Δ* phenotype, suggesting that the Elp3 and Gcn5 proteins have a role in maintaining an enzyme-regulated balance between acetylation and de-acetylation in living cells (Wittschieben et al., 2000).

To support and further extend the above genetic data, the level of histone acetylation in different chromosomal regions was compared in the wild type, *elp3Δ*, *gcn5Δ* and the *elp3Δ gcn5Δ* double mutant by chromatin immunoprecipitation (ChIP) experiments (Kristjuhan et al., 2002; Winkler et al., 2002). When the ChIP analysis was focused on promoters and coding regions of several randomly selected genes it became clear that in the *elp3Δ gcn5Δ* double mutant, the "overall level of acetylation" was dramatically reduced, compared to wild type cells (Kristjuhan et al., 2002).

Elongator binds RNA co-transcriptionally

Elongator was originally isolated as a component of native elongation complexes containing both template DNA and RNA product (Otero et al., 1999). Later on, Winkler et al. reported that Elongator could bind DNA and mononucleosomes *in vitro* (Winkler et al., 2002). More recently, the same complex was shown to bind to RNA both *in*

vitro and *in vivo*. In fact, Elongator, like RNAPII, was found to crosslink along the entire length of nascent, un-spliced pre-mRNA *in vivo* (Gilbert et al., 2004). These results strongly indicated that Elongator plays a role during RNAPII transcription and that it is a component of the RNAPII elongation complex. In Chapter 3 are presented experiments, which demonstrate the role of individual Elongator subunits in RNA-binding *in vivo*.

Genetic interaction between Elongator and other transcription-related proteins

In line with the identified physical association of Elongator with RNAPII (Otero et al., 1999), genetic data have shown that the *ELP3* deletion is lethal when combined with a deletion of the *RPB9* (encoding a non-essential subunit of RNAPII) (Van Mullem et al., 2002). The same was true for the *rpb9Δ gcn5Δ* double mutant cells. This correlates with the proposed model by Wittschieben et al. and Kristjuhan et al. for an overlapping role between Elp3 and Gcn5 (Kristjuhan et al., 2002; Wittschieben et al., 2000).

Moreover, synthetic lethal phenotypes were also observed in *elp3Δ ctk1Δ* (*CTK1* encodes a subunit of the RNAPII C-terminal domain kinase, Ctdk1) (Jona et al., 2001), or *elp3Δ fcp1Δ* double mutant cells (*FCP1* encodes the RNAPII CTD phosphatase) (Stephanie E. Kong, 2004). Finally, synthetic slow growth phenotypes are conferred upon combination of *ELP* deletions with deletions of genes encoding subunits of Mediator, the Paf complex, the Spt16 subunit of the general histone chaperone FACT, and histone modifiers such as the Sin3/Rpd3 histone de-acetylase and the Rad6 histone ubiquitylating enzyme (Formosa et al., 2002; Stephanie E. Kong, 2004).

Though the significance of these interactions is unknown, they support a function of Elongator complex in transcript elongation and chromatin modification.

Human Elongator complex

Following the purification of the human homologues for Elp1 and Elp3, two independent studies provided biochemical evidence for the existence of a human Elongator complex (Hawkes et al., 2002; Kim et al., 2002). In both cases, a three-subunit core complex, including human Elp1 (IKAP), hElp3 and the human homologue of yeast Elp2, was identified. However, when Hawkes et al. utilized an alternative purification scheme in order to avoid anion-exchange chromatography (previously shown to disrupt yeast Elongator), a larger six-subunit Elongator was purified. The three additional proteins had molecular sizes comparable to the yeast Elp4, Elp5 and Elp6 proteins. Mass spectrometry analysis has confirmed that these subunits are indeed the human homologues of yeast Elp4, Elp5 and Elp6 (Hawkes et al., 2002; Svejstrup lab, unpublished data). Similar to yeast Elongator, human six-subunit Elongator, but not core Elongator (composed of only hElp1, hElp2 and hElp3), had HAT activity directed against the tails of histone H3 and H4 (Hawkes et al., 2002).

The two studies also provided compelling, yet contradictory, evidence on the sub-cellular localization of hElongator (Hawkes et al., 2002; Kim et al., 2002). Hawkes et al. detected IKAP and hElp3 proteins predominantly in the nucleus of HeLa cells. Some staining was also detected in the nucleoli and the cytoplasm. In contrast, Kim et al. detected the majority of these proteins in the cytoplasm, and only a minor fraction in the nucleus. The reason for the dramatic

differences remains unclear. It should also be mentioned that the localization studies that have been published for yeast Elongator complex also suggest that the majority of the Elp proteins is found in the cytoplasm under normal growth conditions (Fichtner et al., 2002b; Pokholok et al., 2002). In conclusion, Elongator is believed to be predominantly cytoplasmic in most cell types, in spite of having an important role in nuclear processes and being present in this compartment as well. The cytoplasmic role of Elongator remains unknown.

Consistent with data obtained with the yeast complex, hElongator also interacts directly with purified RNAPII (Hawkes et al., 2002; Kim et al., 2002). Additionally, purified hElongator was found in elongation complexes formed *in vitro*. Moreover, using an *in vitro* reconstituted chromatin template, Kim et al. (Kim et al., 2002) showed that hElongator stimulates transcription through nucleosomes in an acetyl-CoA-dependent manner, but has no effect on transcription on naked DNA.

As mentioned above, the human homologue of the yeast Elp1 protein is the previously identified IKAP protein. This protein has been proposed to be a modulator of different signalling pathways, such as NF-κB- and c-Jun N-terminal kinase-dependent pathways (Holmberg et al., 2002; Krappmann et al., 2000). However, it is currently unknown whether IKAP alone or in the context of the Elongator complex has these functions.

Interestingly, mutations in the *IKBKAP* gene, encoding IKAP, have been shown to be responsible for the autosomal recessive human disease familial dysautonomia (FD) (Anderson et al., 2001; Slaugenhaupt et al., 2001). In this disease there is a major FD

haplotype, which is the result of a truncated IKAP protein (caused by a splice site mutation), and a minor FD haplotype, which disrupts a consensus phosphorylation site in IKAP. Finally, the mouse homologue of the yeast Elp2 protein is the Stat3-interacting protein (StIP1), which was found to regulate the ligand-dependent translocation of Stat3 into the nucleus (Collum et al., 2000). Again it is unknown whether the other Elongator subunits have any roles in the above functions attributed to IKAP and hElp2. From the studies reviewed in this section, it is clear that the potentially multiple roles of Elongator (or at least Elp proteins) still remain poorly understood.

1.3 *Zymocin*

In competing for limited resources, microorganisms have evolved sophisticated strategies to gain selective advantages over their competitors. One of these is the secretion of toxic compounds that results in killing or growth arrest of other species or genera. Yeast *Kluyveromyces lactis* secretes a toxin, referred to as zymocin, which inhibits cellular growth of various sensitive yeast genera, including *S. cerevisiae* (Frohloff et al., 2001). Fluorescence-activated cell sorter (FACS) analyses revealed that upon encountering zymocin, budding yeast cells arrest at the un-budded G1 phase of cell cycle with an un-replicated (1n) DNA content. The exact way the toxin works is not yet known. The initial thoughts that zymocin functions either by blocking the production of cAMP, which is essential for mitotic growth and cell division, or by antagonizing G1 cyclin function, were ruled out (Frohloff et al., 2001). Nevertheless, further evidence that zymocin might act in late G1 before START was provided by the finding that cells, which have been chemically arrested in S phase by

hydroxyurea prior to zymocin treatment, are able to complete one round of cell division and get arrested in the new un-budded G1 cell cycle stage when released from the chemical S block in the continued presence of zymocin (Frohloff et al., 2001).

The native toxin is a heterotrimeric ($\alpha\beta\gamma$) structure composed of three subunits (Jablonowski et al., 2001b). Cytotoxicity resides solely within the γ subunit. Intracellular expression of this subunit alone in *S. cerevisiae* cells is lethal. The α subunit, which exhibits an exo-chitinase activity *in vitro*, and the β subunit, predicted to associate with the cell membrane, are required for native zymocin to act from the cell's exterior by docking to the cell wall-associated chitin and mediating γ-toxin translocation.

Elongator is linked to zymocin function

Genetic screening for mutations that confer resistance towards the intracellular expression of γ subunit of zymocin identified seven genes that were named *TOT1-7* (*Toxin Target*) (Frohloff et al., 2001; Jablonowski et al., 2001c; Otero et al., 1999). Interestingly, among these were all the genes that encode the subunits of yeast Elongator complex (Elp1/Tot1, Elp2/Tot2, Elp3/Tot3, Elp4/Tot7, Elp5/Tot5, Elp6/Tot6) and the Elongator-related Kti12 protein (Tot4). Deletion of any of these genes also confers resistance towards the holo-toxin secreted from *K. lactis* cells. Interestingly, point mutations in Elp3, which abolish its HAT activity, also confer toxin resistance. Moreover, mutagenesis studies on the *ELP3* gene showed that there are mutations outside the HAT domain, which confer sensitivity to killer toxin, but not the so-called "Elongator" phenotype (separation of function mutations) (Jablonowski et al., 2001c). This suggested that

the requirement of Elongator for γ-toxin sensitivity could be genetically dissociated from general Elongator function. Therefore, it was proposed that although the HAT activity of Elp3 is essential for zymocin to act, it is not *per se* sufficient for sensitivity (Jablonowski et al., 2001c). The deletion of other HAT-encoding genes, like *SAS3, HPA3, HAT1* and *GCN5* did not confer zymocin resistance, further suggesting that Elp3 itself and not just any HAT activity is the target of the toxin (Kitamoto et al., 2002).

Effects of mutations in transcription-related genes on zymocin sensitivity

In contrast to deletions of *ELP*, which conferred resistance towards zymocin, deletion of most genes encoding subunits of other transcription-related complexes rendered cells even more sensitive (Kitamoto et al., 2002). This is true for all the genes, which encode subunits of the SAGA, the SWI/SNF, the Mediator and the Ccr4-Not complex. Zymocin hypersensitivity is also observed in cells carrying deletions of transcription elongation-related factors, such as Ctk1, Fcp1, and Rtf1 or mutants of Rpb2. In contrast, histone de-acetylase (HDAC)-defective cells displayed either wild type or even reduced zymocin sensitivity. Based on this Kitamoto et al. suggested that situations which favour histone hyper-acetylation reduce the cells' need for the HAT activity of Elongator and therefore reduce zymocin toxicity (Kitamoto et al., 2002).

Zymocin targets the RNA Polymerase II *complex*

The physical association of Elongator, which is a primary target of zymocin, with RNA Polymerase II prompted the Schaffrath

laboratory to study the effect of the toxin on RNAPII-dependent transcription. Low resolution hybridization showed that global poly $(A)^+$ mRNA levels decline in the presence of zymocin, and Northern blot analysis showed significantly reduced levels of certain RNA Polymerase II-dependent transcripts (*SIC1*, *CLN3*, *HHT1* and *ACT1*) upon zymocin treatment (Frohloff et al., 2001; Jablonowski and Schaffrath, 2002). The same studies suggested that the presence of zymocin correlates with a reduction in the total cellular amount of the un-phosphorylated form of RNA Polymerase II. Chromatin immunoprecipitation experiments suggested that RNAPII became stalled in the early coding region of transcribed genes in a toxin-dependent manner (Jablonowski and Schaffrath, 2002). Preliminary experiments that will be presented in Chapter 4 focus on the effect of zymocin on RNA Polymerase II protein levels.

In an attempt to reinforce the notion that zymocin action is linked to a functional RNA Polymerase II, the Schaffrath laboratory studied the effects on zymocin toxicity of genetic conditions known or assumed to directly impair RNAPII activity, such as mutations in the RNAPII kinase encoded by the *BUR1/BUR2* genes, deletion of the *CTK1*, which encodes the α-subunit of the CTDK-I kinase, deletion of the *SRB10* CTD kinase gene and inactivation of the kinase activity of TFIIH (Jablonowski and Schaffrath, 2002). In all these cases the cells exhibited hypersensitivity towards zymocin. Moreover, hypersensitivity was caused not only by the impairment of RNA Polymerase II function by (partial) loss of CTD-kinase activities, but also by truncation of the CTD itself, further supporting the idea of a functional link between zymocin and the CTD of RNA Polymerase II (Jablonowski and Schaffrath, 2002).

Kti12: a new Elongator subunit?

Apart from *ELP*, *KTI12* was also identified from the genetic screen for targets of the toxin zymocin. Cells lacking this gene displayed slightly slower growth rates under normal conditions, very slow growth at high temperature (39°C) and in the presence of the transcription elongation-specific inhibitor 6-azauracil (6-AU), as well as hypersensitivity to Calcoflour White (indicative of cell wall defect), and to caffeine (Fichtner et al., 2002a). Thus, *kti12Δ* cells display the "Elongator" phenotype that was described earlier for *elpΔ* mutant cells.

Database searches with the *KTI12* revealed significant homology with proteins from human, mouse, *Arabidopsis*, *Drosophila*, fission yeast, *C. elegans* and archaea, suggesting that Kti12 (like the Elongator subunit Elp3), is a universal and ancient protein (Fichtner et al., 2002a; Nelissen et al., 2003). Regions of highest homology are located within the amino-termini, including a P-loop motif, which is a conserved ATP/GTP binding domain. This function seems to be essential for the protein, since a *S. cerevisiae* amino-terminally truncated Kti12 variant devoid of the P-loop motif failed to complement the zymocin resistant phenotype associated with *kti12Δ* cells.

The similar phenotypes observed for the *S. cerevisiae elpΔ* and *kti12Δ* cells prompted the Schaffrath laboratory to study possible physical interactions between these proteins. Low stringency co-immunoprecipitation experiments indicated interaction of Kti12 with Elongator and with the serine 5 phosphorylated form of RNAP II (Jablonowski and Schaffrath, 2002). However, deletion of *KTI12* did not affect the structural integrity of six-subunit Elongator complex,

making it unlikely that Kti12 is a structural component of Elongator. Although, co-immunoprecipitation experiments were utilized to map the interactions of Kti12 with Elongator proteins, no firm conclusions were reported for direct interactions (Fichtner et al., 2002b; Frohloff et al., 2001). Therefore, it was obvious that the exact nature of the physical and functional interaction between Elongator and Kti12 remained to be established (see Chapter 4).

Immunolocalization studies suggested that, like Elongator, Kti12 is present mainly in the cytoplasm (Fichtner et al., 2002b). In the same study the authors also presented low quality ChIP data suggesting that Kti12 might occupy the promoter but not the coding region of the *ADH1*. Interestingly, this promoter recruitment appeared to be enhanced in the presence of zymocin (Fichtner et al., 2002a). However, data, which will be presented in Chapter 4, argue against the presence of Kti12 only at the promoter of the *ADH1* gene.

Model for a functional interplay between Elongator, Kti11, Kti12 and Sit4 in zymocin mode of action

Three recent reports have focused on the role of proteins thought to interact physically and/or functionally with Elongator in regulating zymocin's mode of action (Fichtner et al., 2003; Jablonowski et al., 2001a; Jablonowski et al., 2004). One of these proteins is called Kti11 (Killer toxin insensitive 11). TAP-tag purification of this protein revealed an interaction with the three largest subunits of Elongator, Elp1, Elp2 and Elp3 (Fichtner et al., 2003). Surprisingly, the same laboratory previously reported that they could not co-immunoprecipitate any Elongator subunit with the Kti11 protein (Fichtner and Schaffrath, 2002). Therefore, the value

of the data on co-purification must be considered questionable, until a functional relationship between the two proteins is presented. The authors also reported that the Kti11 protein is dispensable for Elongator complex formation. However, they noticed that the Elp1 protein that co-purified with Kti11 migrated in three distinct forms in a SDS-PAGE (Fichtner et al., 2003). Interestingly, the pattern of Elp1 PAGE migration changed in extracts from *KTI11* deletion mutants so that the most prominent form was now the faster migrating. Mass spectrometry analysis showed that the two faster migrating forms were truncations of the full length Elp1 protein (the slower migrating form). Thus, the authors suggested that Elp1 might be prone to proteolysis, which is normally suppressed by Kti11, but failed to demonstrate any functional significance for the presence of truncated Elp1 proteins (Fichtner et al., 2003). Studies in the Svejstrup laboratory are now under way to establish the physical and functional interaction between Elongator and Kti11.

Genetic data from the Schaffrath laboratory also suggested a role for the protein phosphatase Sit4 in the mechanism underlying zymocin function (Jablonowski et al., 2001a). Deletion of *SIT4* conferred resistance to the toxin. The same was true for a double mutant, which lacked the genes for two activators of Sit4 function (Sap185, 190 (Sit4-associated protein)), suggesting a role for the Sit4/Sap185/Sap190 complex in zymocin action. Given that mutations in the *ELP* genes also confer resistance towards zymocin and that *sit4Δ* or *sap185Δ sap190Δ* double mutant cells share some phenotypes with *elpΔ* mutants, such as high temperature sensitivity and sensitivity to 6-AU (6-azauracil), Jablonowski et al. studied a possible functional link between Sit4 and Elongator (Jablonowski et al., 2004).

Although, Sit4 was found not to play any apparent role in Elongator gene expression, assembly or integrity, it was suggested that it might be crucial for de-phosporylation of Elp1 (Jablonowski et al., 2004). In *sit4Δ* cells the equilibrium between phosphorylated and un-phosphorylated Elp1 was shifted towards the former. In contrast, cells lacking either *KTI11* or *KTI12* had no phosphorylated form of Elp1, suggesting that the Sit4 and the Kti12/Kti11 proteins have antagonistic effects on the phosphorylation of Elp1. Based on the above and other observations, Jablonowski et al. proposed a model according to which de-phosphorylation of Elp1 by Sit4 is needed for G1 exit. In the presence of zymocin, sequestration of Elongator prevents START execution and G1 exit (Jablonowski et al., 2004). However, no direct evidence has been presented for a functional significance of the phosphorylation of Elp1 and a cell-cycle dependent role of Elongator complex.

As should be obvious from the text above, a large and somewhat confusing body of recent data has implicated a number of different proteins (Kti12, Kti11, and Sit4) with the structural integrity and/or the role of Elongator in zymocin action. However, very little is known about the real effect of these proteins on Elongator. Studies that will be presented in Chapter 4 and others, which are currently under way in the Svejstrup laboratory, are focused on providing better evidence of these effects.

1.4 ***Aims of the thesis***

The main focus of this study was to provide an outline of the molecular architecture of the Elongator complex, identify roles for its individual subunits, and uncover the physical and functional

relationship between this complex and the Kti12 protein. Additionally, the role of Elongator, Kti12 and Def1 protein in the mechanism of zymocin action is studied.

Key questions that will be addressed in the next Chapters include: what are the roles of the individual Elongator proteins in the integrity of the complex and the functions that are attributed to it? Does Elongator shuttles continuously from the cytoplasm to the nucleus and back? Is there a physical interaction between Elongator and Kti12 and what are the functional consequences of this interaction? Is there an overlapping role for Kti12 and Elongator *in vivo* other than the mediation of zymocin function? Does zymocin function lead to degradation of RNA Polymerase II?

CHAPTER 2

MATERIALS AND METHODS

Many of the methods given here are taken, or adapted from Sambrook et al., 1989 (Sambrook J, 1989).

2.1 Commonly used buffers and solutions

Materials used were generally obtained from Sigma and were of the highest purity available, unless otherwise stated.

10x Blue Juice: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in deionised water (dH_2O).

10x Gel running Buffer (GRB): 0.25 M Tris base, 1.92 M glycine, 35 mM SDS.

3x Laemmli sample buffer (Laemmli buffer): 20% glycerol, 2% ß-mercaptoethanol, 9% SDS, 0.1875 M Tris-.HCl pH 6.8, 0.0125% bromophenol blue.

LB: 0.5% bacto-tryptone, 0.25% bacto-yeast extract, 170 mM NaCl, pH to 7.0 with NaOH. Sterilise by autoclaving.

LB/Amp: LB with 100 µg/ml ampicillin.

PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH to 7.4 with HCl. Sterilise by autoclaving.

PBST: PBS with 0.02% Tween20.

Ponceau S stain: 1.3 mM Ponceau S, 30% (w/v) Trichloroacetic acid, 118 mM Sulphosalicylic acid.

5x TBE: 0.45 M Tris base, 0.45 M boric acid, 10 mM EDTA (pH 8.0).

TE: 10 mM Tris.HCl (pH 8.0), 1 mM EDTA (pH 8.0).

Transfer buffer: 25 mM Tris base, 192 mM glycine, 20% methanol.

YPD: 1% yeast extract, 2% peptone, 2% glucose.

YNB: 0.67% Yeast nitrogen base without amino acids (Difco), 2% glucose, appropriate amino acids at 40 μg/l final concentration.

2.2 General methods

Agarose gels

Agarose gels of 0.7-2% were prepared (Gibco BRL) and run in 1xTBE. Ethidium Bromide was added to the gel to a final concentration of 0.5 μg/ml. Blue Juice was used as loading buffer at a final 1x dilution. Gel running equipment was BioRad mini (50 ml gel), which was run at 80 V until the required separation was achieved. Gels were visualised on a transilluminator (Ultra Violet Products). If necessary, DNA bands were excised from gel and purified using Qiaquik Gel Extraction kit (QIAGEN) according to manufacturer's protocol.

Enzymatic reactions

Restriction digests, ligations and de-phosphorylations were performed with buffers and protocol supplied with the enzymes. Restriction enzymes and T4 DNA ligase were from NEB, calf intestinal alkaline phosphatase was from Boeringher Mannheim. Enzymes and small DNA fragments were removed after reactions with Qiaquik Nucleotide Removal Kit (Qiagen) according to manufacturers protocol.

Oligonucleotide preparation

Oligonucleotides were obtained from the CR-UK synthesis service. The pellet was dissolved in 200 µl oligo buffer (0.3 M sodium acetate, 10 mM magnesium chloride) and 600 µl cold ethanol was added. The solution was placed on dry ice for 15 minutes then centrifuged at 14000 rpm for 20 minutes at 4°C. The pellet was washed in 80% ethanol in H_2O then dried in a speed-vac. The oligo was dissolved in sterile water at a final concentration of 500 pmol/µl.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gels containing 6-10% 37.5:1 acrylamide:bis-acrylamide were cast according to Sambrook et al. in Mini-Protean II gel system (Bio Rad). Alternatively, pre-cast gels from Bio Rad were used according to manufacturer's recommendation. Samples to be loaded were mixed with 3x SDS loading buffer and denatured by incubation at 100°C in a heat block for 5 minutes. Gels were subjected to 200 V in 1x Laemmli buffer until size markers (Broad Range Protein Markers, New England Biolabs) were appropriately separated.

Coomassie staining of protein gels

For Coomassie staining, the gel was transferred into a clean plastic box and Coomassie stain added to cover the gel. This was incubated at room temperature with agitation for 30 minutes. The stain was removed and kept for re-use. The stain was removed by three 30 minute washes in de-staining solution, with the inclusion of a sponge piece to help absorb the excess dye. After the stain was sufficiently removed the gel was

soaked in 5% glycerol in dH_2O before air-drying between 2 porous cellophane sheets (BioRad).

Coomassie Stain: For 1 Litre; 450 ml dH_2O, 450 ml methanol, 100 ml glacial acetic acid, 1 g coomassie blue.

De-staining solution: 10% methanol, 10% acetic acid in dH_2O.

Silver staining of protein gels

After proteins were separated on a SDS-PAGE gel, the gel was fixed in Solution 1 for at least 1 hour and then in Solution 2 for 30 minutes. It was then rinsed for 1 minute in Solution 3 and washed with H_2O three times over 1 minute. The gel was then incubated in Solution 4 for 20-45 minutes before another wash with H_2O, as above. The signal was developed during incubation in Solution 5 until the desired intensity, and the reaction was stopped by incubation in 1% acetic acid for at least 1 hour.

Solution 1

Methanol	50% v/v
Acetic Acid	12% v/v
Formaldehyde	0.05% v/v

Solution 2

Ethanol	50%

Solution 3

$Na_2S_2O_3$	0.02% w/v

Solution 4

$AgNO_2$	0.2% w/v
Formaldehyde	0.075% v/v

Solution 5

Na_2CO_3	6% w/v

$Na_2S_2O_3$ 0.0004% w/v

Formaldehyde 0.05% v/v

Western Blot Analysis

After proteins were separated by SDS-PAGE, they were transferred onto nitrocellulose (Hybond-C, Amersham) using the Mini-Protean II Western system according to manufacturers instruction. The membrane was then blocked for one hour in 5% milk powder (Marvel) in 1x PBS, 0.02% Tween before incubation with primary antibodies for either overnight at 4°C or two hours at room temperature. The blot was then washed three times, 10 minutes in 1x PBS, 0.02% Tween and then probed with secondary antibody for one hour at room temperature. After washing again as above, the signal was visualised with Super Signal West Pico Chemiluminescent Substrate from PIERCE according to manufacturer's instructions. It should be mentioned that all the antibodies (Table 2.1) were diluted in 5% milk in PBST (0.02% Tween).

Protein concentration determination

1 µl sample extract was added to 800 µl dH_2O in an Eppendorf tube, and was mixed by vortex. 200 µl BioRad protein assay reagent was added and mixed. The samples were transferred to plastic cuvettes (Elkay) and the absorbance at 595 nm determined using a Shimadzu spectrophotometer. A standard curve was prepared using this method and known concentrations of bovine serum albumin (BSA). From this, the concentration of protein in the samples was interpolated (Figure 2.1).

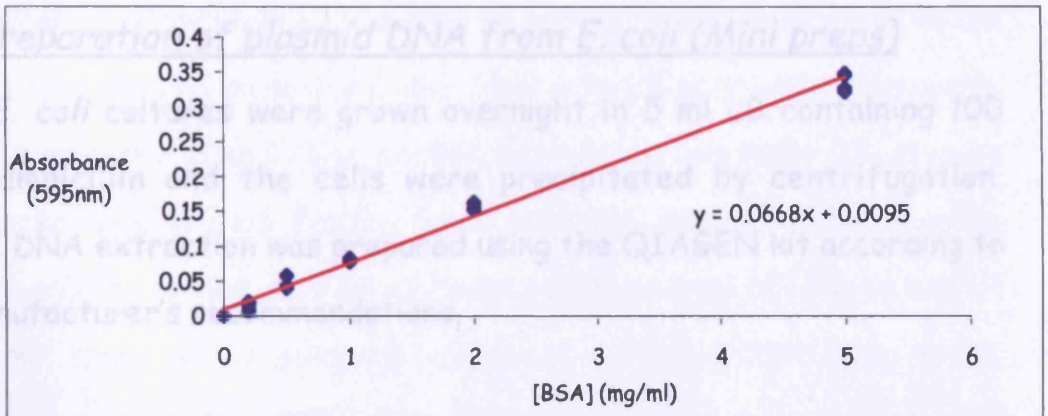

Figure 2.1 Standard curve from bradford protein reagent using known amounts of BSA. The samples were prepared as described above.

Polymerase chain reaction (PCR)

PCR reactions were performed in a DNA Engine (GRI) thermal cycler in 50 µl reaction volumes. Templates were either plasmids or yeast chromosomal DNA, and oligonucleotides were as listed in Table 2.2. The template was denatured for 3 minutes at 95°C and was typically amplified by 25-30 cycles of

		Reaction conditions	
95°C	2 min		
95°C	30 sec	1x PCR buffer	5 µl
52-62°C	1 min	Template	100 ng
72°C	3 min	Oligonucleotides	20 pmoles each
72°C	10 min	dATP, dCTP, dGTP, dTTP (Pharmacia)	0.2 mM each
		PFU polymerase (Roche)	1 unit

Modified conditions were used when necessary.

2.3 *E. coli* manipulation

Mini preparation of plasmid DNA from E. coli (Mini preps)

E. coli cultures were grown overnight in 5 ml LB containing 100 μg/ml ampicillin and the cells were precipitated by centrifugation. Plasmid DNA extraction was prepared using the QIAGEN kit according to the manufacturer's recommendations.

Transformation

An aliquot of competent cells (BL21 DE3 or DH5α Library Efficient Competent Cells from STRATAGENE) thawed on ice. 1-5 μl ligation reaction or miniprep DNA was added to chilled Eppendorf tubes and 100 μl cells were added. After mixing the transformation mixture was incubated on ice for 30 minutes, then at 42°C for 45sec, then re-placed on ice for 2 minutes. 900 μl LB was added and the cells incubated for 30 minutes at 37°C, after which time samples were plated onto LB plates with the addition of the appropriate antibiotic and incubated overnight at 37°C.

2.4 Yeast manipulation

Preparation of media

Yeast cultures were grown in conical flasks in YPD liquid medium at 30°C, with agitation at approximately 200rpm. The volume of culture did not exceed one quarter of the nominal flask volume to ensure adequate aeration. Solid YPD media contained 2% agar. Diluted liquid yeast cultures

were plated for single colonies using 5 to 10 sterile 4mm glass balls (Fisher) per plate. Normally, yeast colonies were streaked out to give single colonies. Plates were incubated at appropriate temperature in the dark. In case of solid selective media, YNB with 2% agar and the required amino acids were mixed. The carbon source was normally 2% glucose unless otherwise stated. Finally, liquid synthetic complete (SC) medium was prepared by mixing 3.35 gr. of Yeast Nitrogen Base (SIGMA), and 1.4 gr. Yeast Drop out Medium Supplement (without histidine, uracil, tryptophan and leucine, SIGMA) in 1 l of water. This was sterilised and the required amino acids and the carbon source was added.

Phenotypic analysis

Growth analysis under the indicated conditions (high salt, 30°C, 37°C, 200 ng/ml LMB) was tested either by streaking or by spotting serial dilutions of the cells on the appropriate medium. To analyze killer toxin sensitivity, *K. lactis* cells expressing or not the toxin zymocin were left to grow o/n on YPD medium, and the next day approximately 10000 *S. cerevisiae* cells, from the indicated strains, dissolved in water and spotted at the vicinity of the *K. lactis*. The growth of the different cell types was compared to the characteristic eclipse growth of wild type *S. cerevisiae* cells.

Transformation

A culture of the strain to be transformed was grown to mig-logarithmic growth phase. $1x10^9$ cells were harvested by centrifugation, washed twice in TE then re-suspended in 1 ml LiAc solution (0.1M

$LiCH_3CO_2$ pH 7.5 in TE). On top of a cell pellete derived from 200 µl of the above suspension were added sequentially 240 µl of PEG (50% Polyethylene glycol MW 3350), 36 µl of 1 M LiAc, 25 µl single stranded herring sperm carrier DNA (2 mg/ml) and 50 µl of a mixture of sterile water and transforming DNA (0.1-10 µg). The cells were re-suspended by vortex and incubated with agitation for 30 minutes at 30°C followed by 20-30 minutes at 42°C. Finally the cells were precipitated by centrifugation at 6000 rpm, re-suspended in 1 ml of sterile TE, and plated directly onto the appropriate selective media.

Genomic DNA preparation

Genomic DNA was prepared from 5 ml yeast cultures in YPD or selective media, grown overnight at 30°C, using the Yeast Genomic Mini Kit from CAMGEN according to the manufacturers protocol.

In vivo tagging of yeast proteins

For the construction of both Elp4-HisHA and Kti12-HisHA strains the C-terminus of the open reading frame of both genes was amplified using the primers that are shown in Table 2.2 and cloned into pSE.HISHA-304, using the Kpn I and BamH I sites to produce plasmid pELP4-HISHA-304, and pKTI12-HISHA-304, respectively. In both cases, after yeast transformation with linearized plasmids, a TRP+ clone was isolated in which the 3' end of either the *ELP4* or the *KTI12* gene was replaced by homologues recombination, resulting in expression of an Elp4-(His)10-HA or Kti12-(His)10-HA fusion protein. The presence of the tag was confirmed by PCR analysis. To ensure that the (His)10-HA

epitope tag did not interfere with Elp4 or Kti12 function, phenotypic analysis was performed.

As far as the construction of strains carrying 18Myc-tagged Elp1, Elp3 and Kti12, and 6HA-tagged Kti12 protein concerns, wild type or any mutant cells were transformed with a PCR product, which includes the C-terminus of the genes that encode the above proteins and the multiple epitopes, using the primers that are shown in Table 2.2. This PCR product replaces the endogenous C-terminal region of these genes by homologous recombination. The plasmids that were used as a substrate for the PCR were kindly provided by Dr. Kim Nasmyth (Knop et al., 1999). Details are available on request.

Finally, the GFP-tagged Elp3 strain that was used was constructed based on the study from Longtine et al. (Longtine et al., 1998). The plasmid that was used as a substrate for the PCR was a kind gift from Dr. Akash Gunjan (Chromosome Dynamics Lab, Clare Hall, CR-UK). The strain *xpo1-1* transformed with plasmids pKW457 and pKW430 was kindly offered from Dr. John F. Diffley (Chromosome Replication Lab, Clare Hall, CR-UK) (Stade et al., 1997). Dr. J. F. Diffley very kindly also provided the strain *crm1Δ* CRM1T539C, with the permission from Dr. M. Rosbash (Neville and Rosbash, 1999).

Two hybrid interactions

The coding sequence of all the six *ELP* genes was cloned in both 'GAL4-activation domain'- and 'GAL4- DNA binding domain'-based vectors provided from the GAL4 Two-Hybrid Phagemid Vector Kit (Stratagene), using a combination of PCR amplification and sub-cloning from other

vectors. In order to detect interactions, the plasmids were introduced in genetically modified yeast cells provided from the Matchmaker Library Construction & Screening Kit (Clontech). The interaction studies were done according to the manufacturer's recommendations.

Fluorescent activated cell sorting (FACS)

$1x10^7$ cells were collected for each sample, re-suspended in 70% ethanol, pre-chilled at -20°C and incubated for 30min at room temperature (cells could stay at this stage for several days at either room temperature or 4°C). When cells were ready for analysis they were washed twice with 1 ml 50 mM Tris-HCl pH 7.8, before they were re-suspended in the same buffer containing 10 μl of RNAse A (SIGMA, 10 mgr/ml). After 2-4 hrs incubation at 37°C, cells were precipitated by centrifugation and re-suspended in 500 μl FACS buffer (200 mM Tris-HCl pH 7.5, 200 mM sodium chloride and 78 mM $MgCl_2$) with the addition of 15 μl propidium iodide (1 mgr/ml, SIGMA). At this stage cells could also be stored at 4°C. Just before analysis, cells were sonicated for 10sec. Fluorescence from intercalated propidium iodide is measured by FACScan (Beckton-Dickinson).

Zymocin treatment of S. cerevisiae cells

K. lactis cells were grown in rich medium for 2 days, harvested and 200 ml of cell-free supernatant was mixed with 200 ml of *S. cerevisiae* cells grown at mid-log phase, at a final concentration of $0.5\text{-}1x10^6$ cells/ml. The *S. cerevisiae* cells were then left to grow for up to 6 hours after the mixing.

2.5 Protein extraction

Native insect extracts

SF9 insect cells were transformed and processed according to the Bac-N-Blue baculovirus protocol (Invitrogen). For the production of crude protein extracts the transformed cells were harvested with a mild spin (2000 rpm, 5min, 4°C), washed first with PBS and then with Hypotonic buffer (20 mM Hepes (KOH) pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$). After incubation on ice with the same buffer for 20min, cells were broken using a B pestle Dounce homogenizer. To separate the cytosolic extract from nuclear pellet the broken cells were spun at 10000 rpm for 10min, 4°C.

Denatured E. coli extracts

1 ml of *E. coli* cells was centrifuged at 14000rpm at room temperature for 1 minute. The cell pellet was re-suspended in 100 µl 3x Laemmli buffer per 1 unit of absorbance at 600nm. This was boiled for 5 minutes, centrifuged and the supernatant retained. 10 µl of this extract was sufficient for visualising by Coomassie staining after SDS-PAGE.

Expression of recombinant GST and GST-Elp5 proteins in bacteria

In order to generate a GST-Elp5 fusion protein, the ORF of the *ELP5* gene was cloned in the pGEX-3X vector (Amersham Bioscience), and the plasmid introduced in BL21 DE3 competent bacteria cells. Cells were grown at 37°C and induced with 1 mM IPTG for 3 hours. After cell re-suspension in PBS (Phosphate buffer saline), lysozyme (50 mg/ml) was

added, and the cell suspension was incubated for 30 min on ice, and then subjected to sonication. The extract was incubated for 1 hour at 4°C with Triton X-100 in a final concentration of 1%, and the soluble supernatant was collected after a 10 min, 12000 rpm spin at 4°C. This soluble supernatant contained GST-Elp5, which was purified and immobilized on glutathione beads (Amersham Pharmacia) by incubation for 30 min at room temperature. The soluble GST control protein was produced in the same way, based on the expression from pGEX-3X vector.

Generation of an anti-Kti12 antibody (Max Soegaard)

The Kti12 ORF was cloned in frame with the GST protein in pGEX-3X and the fusion protein expressed in BL21 DE3 by induction with 1 mM IPTG for 6 hours at 28°C. Subsequently, Max Soegaard, another PhD student in the laboratory, prepared and purified the GST-Kti12 fusion protein. Cells were lysed in PBS by french press and inclusion bodies were solubilised by treatment of the pellet with 0.5 % Sarcosyl and sonication. GST-Kti12 was purified on glutathione-sepharose (Amersham Biosciences) as described above. The recombinant fusion protein was used to immunize rabbits (Imgenex). The resulting antibody was used for Western blots at 1:1000 final dilution.

Rapid yeast whole cell extract preparation

Whole cell extracts were prepared as was described elsewhere, from the indicated strains (Kushnirov, 2000).

2.6 Biochemical methods

Antibody coupling to Protein A beads

400 µl of 50% protein A slurry (Pharmacia) were re-suspended in 15 ml PBS, centrifuged at 3000rpm and washed a further two times in PBS. The beads were then transferred to a 1.5 ml Eppendorf tube. 0.625 ml purified 12CA5 antibody solution (stock concentration 1.6 mg/ml, a final ratio of 5 mg antibody to 1 ml beads) was added and binding allowed for 2 hrs at room temperature with agitation. After centrifugation at 3000rpm the supernatant was discarded and the beads washed into 15 ml PBS, and then with a further 2x 15 ml PBS. A borate buffer was prepared from 0.1 M boric acid and 0.1 M sodium borate solutions, which were mixed in a ratio of 7:4, to give a buffer with pH 9.0. The beads were washed twice in 15 ml of this buffer. 0.5 g dimethylpimedilate (stored desiccated at -20°C) was dissolved in 50 ml 0.1 M sodium borate. The resulting solution was checked to ensure the pH was above 8.5, and then 15 ml was added to the washed beads and coupling was allowed to occur for 3 hour at room temperature in a tube fixed onto a rotating wheel. After this time, the beads were pelleted and re-suspended in 25 ml 200 mM Ethanolamine pH 8. This step was repeated once more and then the beads were incubated at room temperature for 4 hours with rocking. Finally the beads were washed three times in PBS and re-suspended in PBS as a 50% slurry. Sodium azide was added at 0.02% final concentration and the beads stored for up to two weeks at 4°C.

In vitro transcription/translation

The recombinant Elp4 and Elp6 proteins used for the *in vitro* pull down experiments were produced using the TnT T7-coupled wheat germ extract systems (Promega) according to the manufacturer's recommendations. The DNA template used was pBluescript II KS (+/-), into which the coding sequence of the two genes was cloned.

In vitro pull down experiments

Immobilized, baculovirus-expressed histidine-tagged Elp1 or Elp2 and bacterially expressed GST or GST-Elp5 proteins were mixed with the product of *in vitro* transcription/translation (Elp4 or Elp6) reactions in a buffer containing 250 mM potassium acetate, 100 mM Hepes-KOH pH 7.6, 20% Glycerol, 0.1% NP40, 1x protease inhibitors and 4 mM ß-Mercaptoethanol and incubated overnight with previously equilibrated in the above buffer Nickel agarose or GST beads. The next day, the beads were washed three times with the same buffer and the bound proteins were separated by PAGE on a 10% SDS gel. The Elp4 and Elp6 proteins showed weak, non-specific binding to Ni-agarose. Therefore, these beads were washed three times with the above buffer but containing 30 mM imidazole pH 7.5

Co-immunoprecipitation experiments

For holo-Elongator co-immunoprecipitation experiments, the yeast protein extracts that were used had previously subjected to Bio-Rex chromatography. 500 µg protein of the appropriate elution fraction adjusted to 500 mM salt in buffer A (see Protein purification) was

incubated with Sepharose A beads, which had been previously conjugated with 12CA5 antibody. The beads were washed three times with the same buffer, re-suspended in 1x SDS loading buffer and the bound proteins were subjected to SDS-PAGE.

Large scale yeast Elongator and Kti12 purification

DNA-free soluble whole cell extract subjected to cation-exchange chromatography on Bio-Rex 70 (Bio-Rad) essentially as described previously (Winkler et al., 2001). Briefly, for every 90 mg of protein to be loaded onto the column, 1 g of Bio-Rex 70 (Bio Rad) was prepared by swelling and washing in Buffer A1200 (40 mM Hepes-KOH pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, 20% (v/v) glycerol containing 1200 mM potassium acetate) three times. The resin was then re-dissolved in A0 (the number following buffer name denotes concentration of potassium acetate in milli Molar units) and equilibrated with 3-5 column volumes of Buffer A150. Protein was loaded onto the column in a buffer of comparable salt concentrations and eluted stepwise with increasing amounts of salt (150 mM, 300 mM, 600 mM, and 1200 mM KOAc), at a rate of 2 column volumes per hour. Fractions were collected in 1/10 column volume volumes and either applied to the next column or quick-frozen in liquid nitrogen and stored at -80°C. The vast majority of Elongator and Kti12 were eluted in the fraction containing 600 mM KOAc. Typically, 50 ml of this fraction was incubated with 0.8 ml of protein A-Sepharose CL4B-12CA5 monoclonal antibody resin (1-3 mg of antibody/ml of resin) overnight at 4 °C. The resin was collected by gravity flow in a column holder and washed extensively with buffer A containing 600 mM

KOAc and equilibrated in A containing 300 mM KOAc. Bound proteins were then eluted in three washes with 1 ml of buffer A containing 300 mM KOAc and 2 mg/ml HA peptide (KKKRILKMYPYDVPDYARIL) for 15 min at 30 °C. These fractions were pooled and subjected to Nickel agarose chromatography as described previously. Bio-Rex 70 resin could be regenerated by a 3-column volume wash of 0.5 M KOH and intensive rinsing with H_2O until the pH dropped to pH 7.5. Then, it can be stored at 4°C in 40 mM HEPES, pH 7.6, 1 mM EDTA, 0.02% sodium azide.

Gel filtration chromatography

Gel filtration analysis was performed using a Superose 6 column connected to a BIOLOGIC FPLC chromatography system (Bio-Rad). The buffer used was 2% glycerol, 250 mM Potassium Acetate pH 7.6, 0.1% NP-40, 1x protease inhibitors. The column was run at a flow rate of 30 µl/min and the protein containing fractions (8-20) were analysed by western blotting. Size markers (Pharmacia) were dissolved in the same buffer and run under the same conditions, immediately before and after each sample.

Nickel Agarose Chromatography

Typically, 0.5 ml Nickel Agarose (Qiagen) was first washed with Buffer H before the protein was loaded. Protein was incubated from 5 hrs to overnight with the resin, which was then washed with 5-10 column volumes of Buffer H. Subsequently, the resin was washed with 5 column volumes of buffer H including 10 mM imidazole, pH 7.5, followed by 5 column volumes of buffer H, 50 mM imidazole, pH 7.5. The affinity column was then eluted first 3 times with 1 column volume of buffer H,

100 mM imidazole, pH 7.5 and then 5 times with 1 column volume of Buffer H, 500 mM imidazole, pH 7.5. All the fractions were quick-frozen under liquid nitrogen and stored at -80°C.

Buffer H

Glycerol	10% (v/v)
HEPES, pH 7.6	40 mM
2-mercaptoethanol	4 mM
Potassium Acetate pH 7.6	300 mM
Triton X-100	0.1%

Protease Inhibitors (final concentrations: 1 mM PMSF, 10 μg/ml Leupeptin, 1 μg/ml Pepstatin A, 10 mM Benzamidine HCl, all from SIGMA)

Chromatin immunoprecipitation assay (ChIP)

Strains were grown in YPD media to a density of 1-1.5 x 10^7 cells per ml and fixed in 1% formaldehyde for 30 min at room temperature. Cells were lysed in FA lysis buffer (50 mM Hepes pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1x protease inhibitors) and chromatin was fragmented by extensive sonication (average fragment size always <500 bp). Whole cell extract from 1 x 10^7 cells was used for immuno-precipitations (incubation at 4° C overnight). Reactions were cleared by centrifugation at maximum speed in a microcentrifuge for 15 min, before 15 μl of Protein A slurry (equilibrated in FA lysis buffer containing 1 mg/ml bovine serum albumin and 1 mg/ml DNA) was added to the supernatant, and the mix was then incubated for 2 hours. Subsequently, the Protein A washed successive with buffers FA,

FA500 (50 mM Hepes pH7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1x protease inhibitors), LiCl (10 mM Tris-HCl, pH 8, 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 1x protease inhibitors) and TE containing 100 mM sodium chloride, each one for 5 min. The beads were then re-suspended in Elution buffer (100 mM Tris-HCl pH 7.8, 10 mM EDTA and 1% SDS-sodium dodecyl sulfate) and incubated for 10 min at 37°C. The elution was repeated once more and the DNA was precipitated by centrifugation after incubation with 2.5 volumes cold 100% Ethanol and 1/10 of volume 3M sodium acetate pH 5 either 1 hr at -80 °C or overnight at -20 °C. The precipitated DNA was re-suspended in TE. 1/30 of immunoprecipitated and 1/20,000 of input DNA was used for analysis using a real-time PCR machine. For the experiments shown, results were normalised according to the amount of input DNA, or the amount of non-specific immunoprecipitated DNA. For real-time PCR experiments the results were analyzed by Microsoft Excel programme.

In vitro histone acetyltransferase assay (HAT assay)

Histone acetyltransferase reactions (30 µl) were carried out in buffer containing 10 mM Hepes-KOH (pH 7.6), 5 mM $MgCl_2$, 10 mM sodium-butyrate, 5 mM DTT, 5% glycerol, 0.25 mg/ml BSA, and 0.25 µCi (1 Ci = 37 GBq) [^{3}H]acetyl-CoA (0.25 mCi/ml, 2-10 Ci/mmol). As a substrate, reactions contained 10 µg of core histones commercially available, or 5 µg synthetic peptide mimicking the first 32 (H2A), 33 (H2B), 28 (H3), or 27 (H4) amino acids of the histone amino-terminal

tails. Reactions were carried out for 45-60 min at 30°C. To quantitate the amount of [^{3}H] acetate transferred onto the substrate, the reactions were stopped onto P81 paper or GF/C glass microfiber filters (Whatman). Filters were washed three times in 50 mM sodium carbonate (pH 9.2), once in ethanol, allowed to dry, and subjected to scintillation counting. Alternatively, reactions were terminated by addition of SDS-sample buffer followed by SDS/PAGE using pre-cast 16.5% Tris-Tricine peptide gels (Bio-Rad). After staining of the histones with Coomassie blue, the gels were dried and subjected to fluorography.

2.7 RNA-based methods

Total RNA extraction from yeast

For the detection of *ELP3* mRNA, total RNA was isolated from an equal number of *S. cerevisiae* cells from the indicated strains, using the RNAeasy mini kit (QIAGEN) according to the manufacturer's recommendations. For the detection of 28S and 18S RNA, the gel was stained with EtBr in a final dilution of 1:10000 in water.

Northern blot analysis

The preparation of a 2% gel for electrophoresis and the transfer of RNAs to a Hybond-N nylon membrane (Amersham) was based on Sambrook et al. (Sambrook J, 1989). The probe was a PCR product from the coding region of *ELP3* gene (Table 2.2), which was labelled using the Ready-to-Go DNA labelling beads from Amersham, according to

manufacturer's recommendations. Finally, hybridization of the membrane with the probe was performed using the ExpressHyb kit from Clontech.

RNA immunoprecipitation assay (RIP)

RNA immunoprecipitation was performed essentially as described for ChIP (Kristjuhan et al., 2002), but with some important modifications. Briefly, cells were fixed in 1% formaldehyde for 30 min at room temperature, lysed in FA lysis buffer (50 mM Hepes-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, protease inhibitors) containing RNAse inhibitor (RNASIN (Promega)); 50 U/500 µl buffer), and then nucleic acids were fragmented by extensive sonication. Before immunoprecipitation, the extract was treated with DNAse (400-500 units RNAse-free DNAse I (Sigma) per 500 µl extract) in 25 mM $MgCl_2$, 5 mM $CaCl_2$, 3 µl RNASIN for 10 min at 37°C. Nuclease digestion was stopped by the addition of EDTA to 20 mM. Whole cell extract from 1-2 x 10^8 cells was typically used for immuno-precipitations, but this varied depending on the abundance of the transcript to be probed for. Immuno-precipitation was performed by adding the relevant antibody to the DNAse-treated extract and incubating at 4° C overnight. Reactions were cleared by centrifugation at maximum speed in a microcentrifuge for 15 min, before 15 µl of Protein A slurry (equilibrated in FA lysis buffer containing 1 mg/ml bovine serum albumin and RNAse inhibitor) was added to the supernatant, and the mix was then incubated for 2 hours. All subsequent precipitate washes and the final elution were performed as for ChIP (Kristjuhan et al., 2002), except that buffers contained RNAse inhibitor (50 U RNASIN/500 µl buffer). The eluate

fraction was adjusted to 200 mM NaCl, before addition of 20 μg Proteinase K and incubation at 42°C for 1 hr and then at 65° for 5 hrs to overnight. The reactions were extracted with an equal volume of acid-equilibrated (pH 4.8) phenol:chloroform 5:1. Sodium acetate, pH 5 at final concentration 0.3 M, 20 μg glycogen and ice-cold absolute ethanol at final concentration ~70% were added to the aqueous layer before precipitation at -80° for 1-2 hrs. The reaction was centrifuged at maximum speed in a microcentrifuge for 15 min and the precipitate washed with ice-cold 70% Ethanol. RT-PCR was typically performed on freshly prepared RNA using either a one-step reaction protocol as instructed by the manufacturer (Roche 'Titan' kit) or a real-time PCR machine. Anti-Myc antibody 9E10 (or 9E11) was used to precipitate myc-tagged Elp3, or Elp1. RT-PCR samples were separated on a 6% acrylamide/bis (19:1) TBE gel and visualized with Sybr Green (Cambridge Bioscience) at 1/10.000 dilution. For real-time RT-PCR experiments, analysis was carried out using the ABI Prism 7000 Sequence Detection System in combination with TaqMan Reverse Transcription Reagents (Applied Biosystems N808-0234) and the results were processed using the Microsoft Excel programme. In contrast to ChIP, the amount of input RNA required to give a suitable RT-PCR signal (logarithmic range) differs from transcript to transcript in the same RIP assay, because transcript levels can differ dramatically from gene to gene. This often precludes the use of multiplex RT-PCR.

2.8 Microscopy

GFP staining

Living cells were collected by centrifugation at 10,000*g* for 5 s, washed once with PBS re-suspended in a small volume of PBS containing 10 μgr/ml DAPI (SIGMA) and observed microscopically on poly-lysine coated slides. In case of observing fixed cells, the cell pellet was re-suspended in 1 ml of pre-chilled at -20 °C methanol, by vortex and incubated at room temperature for 6min. They then pelleted again as previously and re-suspended in 1 ml pre-chilled acetone, by vortex. The sample could be stored at this point in dark in -20 °C. In order to visualize the sample, cells were spun down and re-suspended again in 20 μl acetone. 10 of those were placed on poly-lysine-coated slides and smeared forth and back across the slide with the side of a pipette tip until dried. 4 μl of DAPI/glycerol (0.3 μgr/ml DAPI in 50% glycerol) were placed on sample and covered with coverslip. At this point the sample is ready to visualized or can be stored at 4°C in dark. Sample images were taken and processed by a combination of Zeiss Axioplan microscope and appropriate filter sets, digital camera (HAMAMATSU C4742-95), and Openlab 2.0.8 application (Improvision).

List of primary Antibodies	Dilution for Western blot
Rabbit Polyclonal Elp1	1:500
Rabbit Polyclonal Elp2	1:250
Rabbit Polyclonal Elp3	1:2000
Rabbit Polyclonal Elp4	1:1000
Rabbit Polyclonal Elp6	1:1000
Rabbit Polyclonal Kti12	1:1000
Rabbit Polyclonal 9110-100 (HA, Abcam)	1:2000
Rabbit Polyclonal Med1 (kind gift from Dr. Bjorklund S.)	1:2000
Mouse Monoclonal 4H8	1:1000
Mouse Monoclonal 8WG16	1:1000
Mouse Monoclonal H14 (Covance)	1:1000
Mouse Monoclonal 9E10 or 9E11	1:1000
Anti-GST (Goat, Amersham Pharmacia)	1:1000
Anti-Tubulin (Rabbit, Oncogene)	1:100

List of secondary Antibodies (SIGMA)	Dilution for Western blot
Anti-mouse IgG	1:10000
Anti-rabbit IgG	1:10000
	(1:20000 for Elp3, 4, 5 and 6 primary Abs)
Anti-goat IgG	1:10000

Table 2.1 List of primary and secondary antibodies used for Western blot

LIST OF OLIGONUCLEOTIDES		
Name	**Sequence (5'→ 3')**	**Use for**
Elp4Ade5'	CATTGTATAACAAATTCGGCTCCCAAA TATCGCATGTACCATGCGGCATCAGAG CAG	*ELP4* K/O
Elp4Ade3'	AAAAGCATGCCGTATATTTCCCATAAA TTGAACCATATTCCTTACGCATCTGTG CGG	*ELP4* K/O
Elp5Kan5'	GCTCCAACCCAAGCTATTGCTACAGGT AGAACAAGATATACAGCTGAAGCTTC GTACGC	*ELP5* K/O
Elp5Kan3'	AATCTGGAAGCACTCACTATTTACCAT CAGAAAGATGAAATACGACTCACTATA GGGAGACC	*ELP5* K/O
Elp6Trp5'	ACCGTCCAGAACCTCCACAAAAATAAC TAAATACACATTTATGCGGCATCAGAG CAG	*ELP6* K/O
Elp6Trp3'	TACGAGAATCAATGTGCCTCGTATATA ATCTTATCATTATCTTACGCATCTGTGC GG	*ELP6* K/O
Elp4His5'	GGCCGGGGTACCGGGGCTGCCGCTGGGA AACTCTG	HisHA tagged Elp4
Elp4His3'	CCGGCCGGATCCATAGTCTAAAGA TATCTTGGTCT	HisHA tagged Elp4

Kti12k/o5'	*GGGTAAAGGGACCTTGTAAAACTTTT GAAAAAACTCCGGTTCTGCTGCTAGT*	*KTI12* K/O
Kti12k/o3'	*CGTCTTGCCATTTACCTTCTGATATTAA TCACATGTATACCTCGAGGCCAGAAGAC*	*KTI12* K/O
GST-Kti12 5'	*CGGTCGACGGATCCAGATGCCACTGGTG CTTTTTACGGG*	GST-tagged Kti12
GST-Kti12 3'	*GCTCGATCCGAATTCAAGTTTTTGTTA AGATAATCAGCG*	GST-tagged Kti12
Kti12HisHA5'	*GGCCTGGGGTACCAGACAACGCTTGCTA AACATTTGGT*	HisHA tagged Kti12
Kti12HisHA3'	*CCGGACCGGATCCATTCAAGTTTTTGTT AAGATAATCAG*	HisHA tagged Kti12
Kti12(6HA)5' or Kti12(18Myc)5'	*CCGCTTTTCGCTGATTATCTTAAGAAAA ACTTGAATTCCGGTTCTGCTGCTAGT*	6HA and 18Myc tagged Kti12
ELP3-pFA6a-F2	*ATGGTAAACTAGGATATGAACTAGACG GTCCATACATGTCGAAAAGAATTCGGA TCCCCGGGTTAATTAA*	GFP-tagged Elp3
ELP3-pFA6a-R1	*CTGCTTGGAAAACCGGCCATGTCGGCG GCACATAAAAGTTCTATTTACCTGAAT TCGAGCTCGTTTAAAC*	GFP tagged Elp3
p138	*ATGCTTCTCGAGATGGCCAGTTCGTCAC ATAAC*	Cloning of *ELP5* in pBS
p139	*TCTAGAGTCGACTTAAAGGGATCCTCAT AATG*	Cloning of *ELP5* in pBS
kap120 5'	*GATGAGCATCCTCAGTCAAGATGAATA AAATAATCTCTCCAAATAAGCCTCCGGT TCTG*	*KAP120* K/O

kap120 3'	*AAAGATACCACTGTAATGTTGTTAGTCGAA CTATATACAATTTTTTACTCCTCGAGGCCAG AAGAC*	*KAP120 K/O*
GAL4BDElp1 5'	*GGCTCAGGAATTCATGGTTGAACATGACAA GAGTGGGTCAAAGAGGC*	Cloning of *ELP1* in two-hybrid vector
GAL4BDElp2 5'	*GGCTCAGGAATTCATGGTGGAATGTATCACT CCCGAAGCCATTTTTA*	Cloning of *ELP2* in two-hybrid vector
GAL4BDElp3 5'	*GGCTCAGGAATTCATGGCTCGTCATGGAAAA GGCCCAAAAACTAACA*	Cloning of *ELP* 3 in two-hybrid vector
GAL4BDELP5 5'	*GGCTCAGGAATTCATGGCCAGTTCGTCACAT AACCCTGTCATTCTTT*	Cloning of *ELP5* in two-hybrid vector
p132	TCTAGAGTCGACTCAAAAATCAACAATATG ACTC	Cloning of *ELP1* in two-hybrid vector
p134	TCTAGAGTCGACCTATTCATATGCTAATGA GTATATAC	Cloning of *ELP2* in two-hybrid vector
p136	ATGCTTCTCGAGATGTCATTTCGTAAAAG AGG	Cloning of *ELP4* in two-hybrid vector

p137	TCTAGAGTCGACTTAATAGTCTAAAGATA TCTTG	Cloning of *ELP4* in two-hybrid vector
p139	TCTAGAGTCGACTTAAAAGGGATCCTCAT ATG	Cloning of *ELP5* in two-hybrid vector
p141	TCTAGAGTCGACTTAGCGATAAAATAGT TTTGTTG	Cloning of *ELP6* in two-hybrid vector
p60EXP. C-t	CGGCCCGTCGACTTAAATTCTTTTCGACA TGTATGG	Cloning of *ELP3* in two-hybrid vector
p60seq1	ATGGCTCGTCATGGAAAAGG	Elp3 probe for Nor-thern blot
p60seq7	GGCTACCACTGCAATACCCGA	Elp3 probe for Nor-thern blot
adh1prom real time fw	TGCTATCAAGTATAAATAGACCTGCAATT	ChIP
adh1prom real time rv	GTGCAGAAAAAGAAACAAGGAAGAA	ChIP
GAL1-D1-REAL -F1	GATCCTTCTGTGTCGGACTGG	RIP
GAL1-D1-REAL -R1	GGAGCACTGGCAAACCTTTC	RIP

adh1 late orf real time fw	TTAGTCGGTGGTCACGAAGGT	ChIP
adh1 late orf real time rv	TTCCAGCCCTTAACGTTTTCA	ChIP
fba1 prom real time fw	GCTTCAATTACGCCCTCACAA	ChIP
fba1 prom real time rv	CCGTTAGACAACATGAGGGATAAA	ChIP
BAT1-REAL -D1-F	TTGGCGACAAAAAATTGGGT	ChIP
BAT1-REAL -D1-R	TGGGCCGAACAACCATAGAT	ChIP
SSA4-REAL-D1-F	TACATTGGAGCCAGTGGAAAAAGTT	ChIP
SSA4-REAL-D1-R	TCACCCGTTAAGATGGCAGCCTGTA	ChIP
CHR4-REAL-F	CTGGCTTAAACCAGCACTATGTATA	ChIP
CHR4-REAL-R	CCAAACAACTATGTAGGACATATTT TTACA	ChIP

Table 2.2 Oligonucleotide sequences

LIST OF YEAST STRAINS

Strain	Genotype	Reference/ Source
S. cerevisiae		
W303-1A	MATa	(Thomas and Rothstein, 1989)
W303-1B	MATα	(Thomas and Rothstein, 1989)
JSY 100	MATα *elp1Δ::LEU2*	(Otero et al., 1999)
JSY 118	MATα *elp2Δ::LEU2*	This study
JSY130	MATα *elp3Δ::LEU2*	(Wittschieben et al., 1999)
JSY672	MATα *elp4Δ::ADE2*	(Winkler et al., 2001)
JSY784	MATα *elp5Δ::KAN*	(Winkler et al., 2001)
JSY786	MATα *elp6Δ::HIS3*	(Winkler et al., 2001)
JSY787	MATα *elp1Δ::LEU2 elp4Δ:: ADE2*	(Winkler et al., 2001)
JSY489	MATa *ELP1(His$_{10}$-HA)::TRP1*	(Winkler et al., 2001)
JSY666	MATa *ELP4(His$_{10}$-HA)::TRP1*	This study
JSY662	MATa *elp2Δ::LEU2 ELP1(His$_{10}$-HA)::TRP1*	This study
JSY964	MATα *elp3Δ::LEU2 ELP1(His$_{10}$-HA)::TRP1*	This study
JSY965	MATa *elp4Δ::ADE2 ELP1(His$_{10}$-HA)::TRP1*	This study
JSY973	MATα *elp5Δ::KAN ELP4(His$_{10}$-HA)::TRP1*	This study
JSY974	MATα *elp6Δ::HIS3 ELP4(His$_{10}$-HA)::TRP1*	This study
JSY754	MATα *ELP1(MYC$_{18}$)::HIS3*	This study
JSY969	MATα *elp3Δ::LEU2 ELP1(MYC$_{18}$)::HIS3*	This study
JSY777	MATα *ELP3(MYC$_{18}$)::HIS3*	(Gilbert et al. 2002)
JSY968	MATα *elp2Δ::LEU2 ELP3(MYC$_{18}$)::HIS3*	This study

JSY975	MATα *elp4Δ::ADE2 ELP3(MYC$_{18}$)::HIS3*	This study
JSY95	MATα *kti12Δ::URA3*	This study
JSY142	MATα *gcn5Δ::HIS3*	(Kristjuhan et al., 2002)
JSY144	MATα *elp3Δ::LEU2 gcn5Δ:: HIS3*	(Kristjuhan et al., 2002)
JSY970	MATα *kti12Δ::URA3 Elp1(MYC$_{18}$)::HIS3*	This study
JSY960	MATα *KTI12(HA$_6$)::HIS3*	This study
JSY993	MATα *KTI12(MYC$_{18}$)::URA3*	This study
JSY996	MATα *KTI12(His$_{10}$-HA)::TRP1*	This study
JSY994	MATα *elp2Δ::LEU2 KTI12(MYC$_{18}$)::URA3*	This study
JSY995	MATα *elp3Δ::LEU2 KTI12(MYC$_{18}$)::URA3*	This study
JSY1001	MATα *kti12Δ::URA3 gcn5Δ::HIS3*	This study
JSY963	MATα *kap120Δ::HIS3 ELP3(GFP):: TRP1*	This study
JSY978	MATα *xpo1Δ::LEU2 ELP3(GFP)::TRP1 + pKW457(xpo1-1)::HIS3*	This study
JSY976	MATα *crm1Δ::LEU2 ELP3(GFP)::TRP1 + pDC-CRM1T539C*	This study
JSY569	MATα *def1Δ::URA3*	(Woudstra et al., 2002)
MGSC 102	MATα *rad26Δ::HIS3*	(Woudstra et al., 2002)
JSY624	MATα *def1Δ::URA3 rad26Δ::HIS3*	(Woudstra et al., 2002)
JSY676	MATα *def1Δ::URA3 elp3Δ::LEU2*	(Reid J., 2004)
JSY991	MATα *kti12Δ::URA3 gcn5Δ::HIS3 elp3Δ::LEU2*	This study

JSY992	MATa *kti12Δ::URA3 gcn5Δ::HIS3 hos2Δ::TRP1 hda1Δ::KAN*	This study
➢	MATa *xpo1Δ::LEU2* + *pKW457(xpo1-1)::HIS3*	(Stade et al., 1997)
➢	MATa *crm1Δ::LEU2* + *pDC-CRM1T539C*	(Neville and Rosbash, 1999)
➢	MATa *rad14Δ::LEU2*	(Woudstra et al., 2002)
➢	MATa *rad1Δ*	Kind gift from Dr. L. Prakash
➢	MATa *rad10Δ*	Kind gift from Dr. L. Prakash

Strain	Mating type	Reference/ Source
K. lactis		
IFO 1267	MATa (toxic)	Kind gift from Prof. Michael J. R. Stark
MBK 801	MATa (non toxic)	Kind gift from Prof. Michael J. R. Stark

Table 2.3 List of yeast strains

CHAPTER 3

YEAST HOLO-ELONGATOR: STRUCTURE-FUNCTION ANALYSIS AND LOCALIZATION STUDIES

3.1 Introduction

Yeast Elongator complex was initially isolated from yeast chromatin as a three subunit complex associated with the hyper-phosphorylated elongating form of RNA polymerase II (Otero et al., 1999). The three proteins were named Elp1, Elp2 and Elp3, respectively, and were also found to form a complex in the absence of RNA polymerase II. Further studies with soluble, chromatin-free fractions from cells expressing a double affinity-tagged Elp1 protein showed that the true form of Elongator is a six-subunit complex, named holo-Elongator. The three additional Elongator proteins were named Elp4, Elp5 and Elp6 (Winkler et al., 2001).

Holo-Elongator can be disrupted into two sub-complexes by anion exchange chromatography (Winkler et al., 2001). Core Elongator consists of Elp1, Elp2 and Elp3 and the small sub-complex consists of the three remaining proteins (Elp4, Elp5 and Elp6). Disruption during chromatography can possibly explain why the small sub-complex was not found associated with the "elongating" form of RNA polymerase II, since the initial purification scheme included a monoQ column (Otero et al., 1999). The six-subunit complex but not the core complex, posses histone acetyl-tranferase activity against mainly histone H3, suggesting that the small sub-complex is indispensable for this function (Winkler et al., 2002). More recently, holo-Elongator was shown to bind RNA *in vitro* and *in vivo* (Gilbert et al., 2004).

Although the above data clearly suggest a role for the Elongator complex in the nucleus, a number of localization studies have created controversy over the last few years. Thus, two independent studies in yeast and one in mammalian cells showed that the majority

of Elongator is found in the cytoplasm, whereas another one clearly showed Elongator to primarily be in the nucleus in mammalian cells (Fichtner et al., 2002; Hawkes et al., 2002; Kim et al., 2002; Pokholok et al., 2002). Finally, a more recent study suggested that yeast Elp1 has a nuclear localization signal, which can locate GFP to the nucleus (Fichtner et al., 2003).

It is well established that a number of transcription factors, from yeast to man, shuttle between these two compartments rather than continuously occupying the nucleus. The first nuclear transport receptor that was identified, capable of both importing and exporting proteins, was the importin/exportin-β family member Crm1 (also called exportin 1/Xpo1p) (Neville and Rosbash, 1999). Crm1 has now been identified as the nuclear export signal (NES) receptor for a variety of proteins, which include transcription factors, kinases and the cytoskeletal component actin (Neville and Rosbash, 1999). These proteins belong to a family of proteins containing leucine-rich NESs. These signals are short sequences (~10 amino acids) with critically spaced hydrophobic residues that are essential for export activity. Crm1-dependent export is the only well studied export mechanism in *S. cerevisiae* (Neville and Rosbash, 1999). Other members of the importin-β family in *S. cerevisiae* are called karyopherins. Like Crm1, these other factors act as receptors for both imported and exported cargoes. A non-essential gene, encoding the Kap120 protein, was shown recently to play a role in mediating the export of the 60S ribosomal subunit (Stage-Zimmermann et al., 2000). Deletion of the *S. cerevisiae KAP120* gene was shown to confer sensitivity towards the toxin zymocin, a phenotype which is also characteristic of *elpΔ* mutants. The interpretation of this result was that Kap120 might play

a role in nucleocytoplasmic distribution of Elongator (Fichtner et al., 2003).

The aim of the experiments described in this chapter was to confirm biochemically and genetically that Elongator exists as six-subunit complex in cells, map important interactions among its subunits, identify possible roles for them in the identified functions of the complex, and explore the possibility that Elp3 actively shuttles from the cytoplasm to the nucleus.

3.2 Purification of holo-Elongator following the Elp4 protein

If Elongator indeed consists of six subunits, it should be possible to isolate this complex by purification of any Elp protein tagged with the epitope used previously for Elp1 (Winkler et al., 2001). Based on the fact that holo-Elongator is composed of two sub-complexes, we considered it relevant to isolate Elongator from cells expressing a tagged version of one of the components of the small sub-complex. A haploid strain expressing a tagged version of the Elp4 protein was therefore constructed. The endogenous chromosomal copy of *ELP4* was modified so that the full-length of Elp4 would be fused to a tag encoding a carboxyl-terminal decahistidine stretch and a HA epitope. A schematic representation of this Elp4 molecule is depicted in Fig. 3.1A. After genetic verification that the tag did not interfere with Elp4 function (these cells, in contrast to *elp4Δ* mutants, were not temperature sensitive), the soluble fraction of whole cell extracts from these cells was isolated. The three step purification scheme that was followed is shown in Fig. 3.1B. The first step was Bio-Rex 70

chromatography. The proteins eluted from this resin with moderate high salt concentration (600 mM potassium acetate) were loaded onto 12CA5-conjugated Sepharose A beads. After overnight incubation and thorough washing, competition with 1 mg/ml HA peptide was used to elute the bound proteins, which were then incubated with Ni^{+}-agarose beads. After washing, the bound proteins were eluted with 500 mM imidazole, yielding purified holo-Elongator (Fig. 3.1B lane 1). As can be seen, however, the three small subunits were super-stoichiometric (Fig. 3.1B compare lane 1 with 3). Interestingly, when the affinity column (HA) was washed with 2 M NaCl, only the three smaller subunits, Elp4, Elp5 and Elp6 co-purified, suggesting that holo-Elongator can be disrupted into two sub-complexes by high salt (lane (2)). Therefore, we can conclude that holo-Elongator behaves biochemically as a fragile six-subunit complex, which is particularly unstable under high salt conditions.

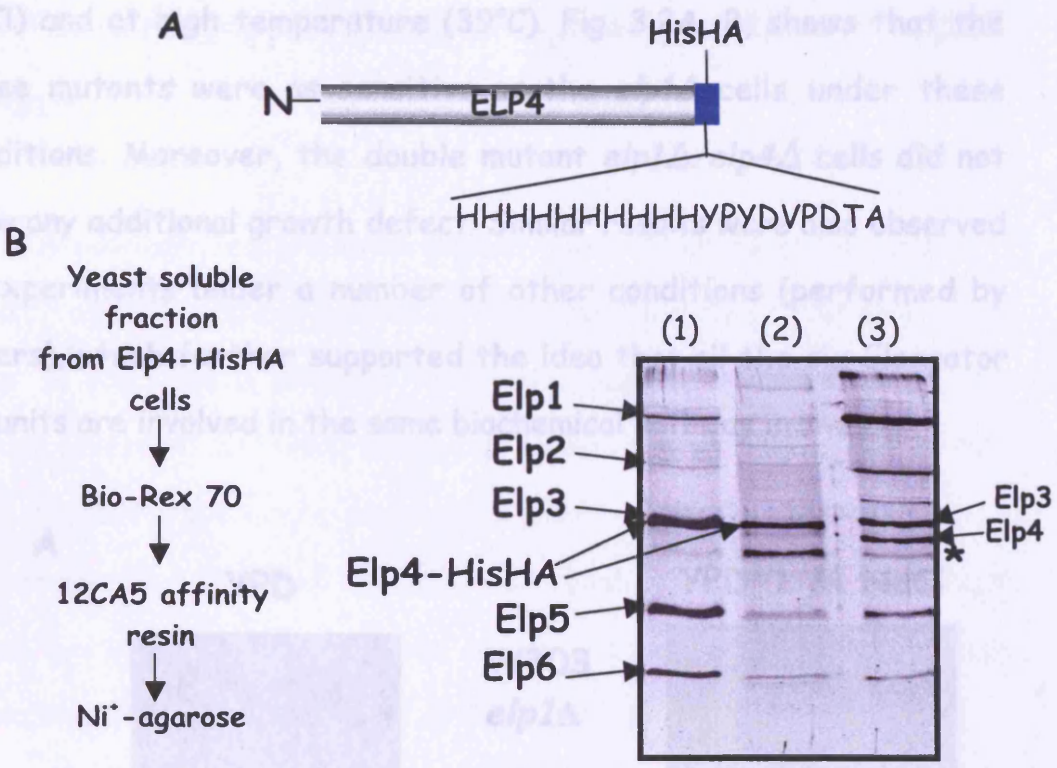

Figure 3.1. Purification of Elp4-HisHA from soluble yeast extract. *A*, Schematic representation of HisHA-tagged Elp4 protein. *B*, Purification scheme and silver staining. Lane (1) is holo-Elongator eluted from Ni^{+}-agarose following the purification of Elp4-HisHA protein. Note that tagged Elp4 migrates at the same position as Elp3. Lane (3) is holo-Elongator obtained from the purification of Elp1-HisHA protein (Winkler et al., 2001) and is used as a control. High salt treatment of Elp4-HisHA bound to 12CA5 affinity resin resulted in disruption of the two sub-complexes (lane (2)). The asterisk indicates an irrelevant contaminant.

3.3 Genetic evidence that holo-Elongator is a six-subunit complex

To investigate genetically whether all the six co-purified subunits function in the same cellular process, phenotypic analysis of cells carrying deletions in the genes encoding the three smaller Elongator subunits was performed. The three deletion mutants were constructed and tested for growth in the presence of high salt (1 M

NaCl) and at high temperature (39°C). Fig. 3.2A, B, shows that the three mutants were as sensitive as the *elp1Δ* cells under these conditions. Moreover, the double mutant *elp1Δ/elp4Δ* cells did not show any additional growth defect. Similar results were also observed in experiments under a number of other conditions (performed by others), which further supported the idea that all the six Elongator subunits are involved in the same biochemical pathway in the cell.

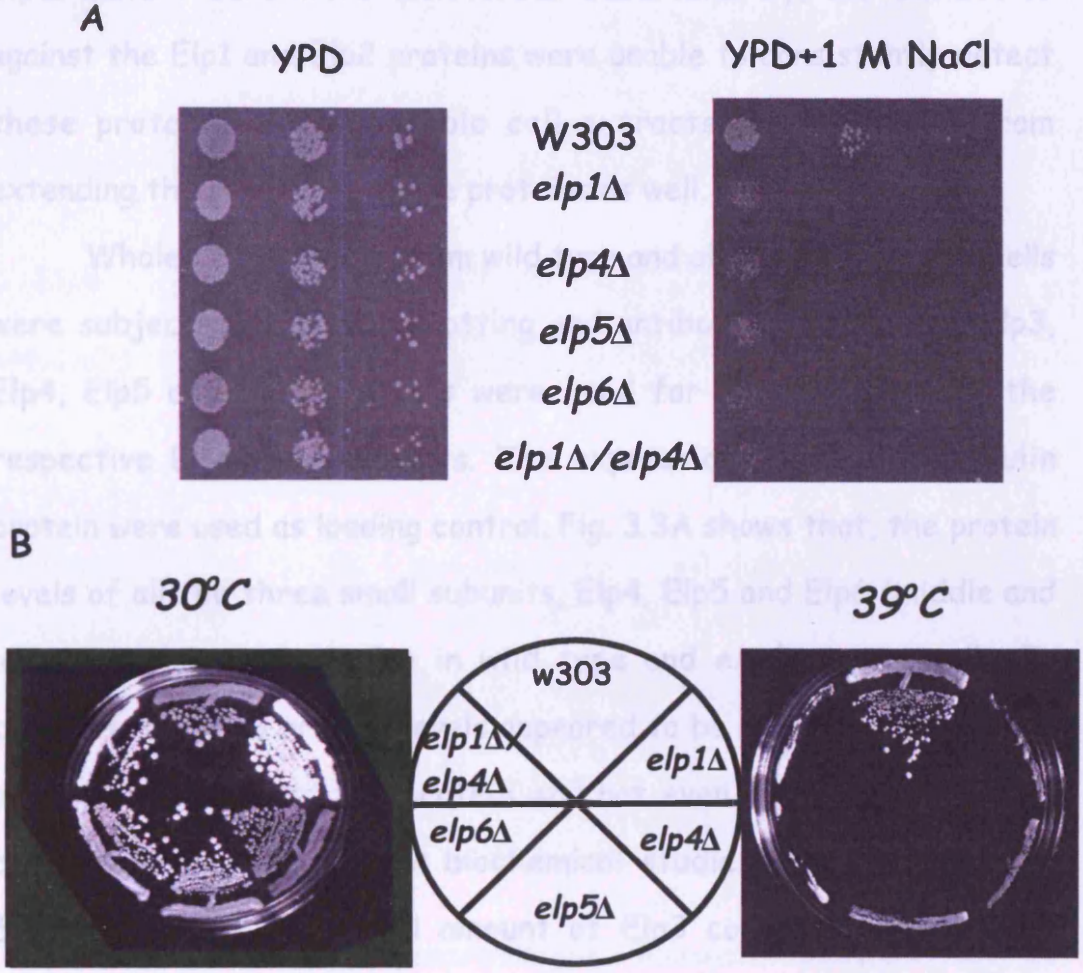

Figure 3.2. Phenotypic analysis of *elpΔ* mutant cells. *A*, Drop test assay to compare the growth of wild type and *elpΔ* mutants in the presence or absence of high salt. *B*, Comparison of growth of wild type and *elpΔ* mutants on YPD plates at 30°C and 39°C.

3.4 The Elp3 protein levels are greatly reduced in *elp1Δ* mutants

A common feature among multi-subunit complexes is that the absence of one of the subunits affects the stability of the others. In order to investigate whether this also applies to holo-Elongator, the protein levels of different Elongator subunits in wild type and all the *elpΔ* mutant cells were monitored. Unfortunately, our antibodies against the Elp1 and Elp2 proteins were unable to consistently detect these proteins in yeast whole cell extracts, precluding us from extending the studies to these proteins as well.

Whole cell extracts from wild type and all the *elpΔ* mutant cells were subjected to immunoblotting and antibodies against the Elp3, Elp4, Elp5 and Elp6 proteins were used for the detection of the respective Elongator subunits. The expression levels of α-tubulin protein were used as loading control. Fig. 3.3A shows that, the protein levels of all the three small subunits, Elp4, Elp5 and Elp6 (middle and lower panels) were similar in wild type and *elpΔ* mutant cells. In contrast, the Elp3 protein levels appeared to be somewhat reduced in all *elpΔ* mutants and the protein was not even detectable in *elp1Δ* cells. However, independent biochemical studies from cells lacking *ELP1* showed that a small amount of Elp3 could be detected by immunoblotting analysis after several steps of concentration and purification (Max Soegaard, PhD student in the lab), suggesting that very small amounts of Elp3 may remain in those cells. Northern blot analysis revealed that the *ELP3* gene is normally transcribed in mutant *elpΔ* cells, apart from *elp3Δ*, as expected (Fig. 3.3B). This indicated that the stability of Elp3 protein requires Elp1.

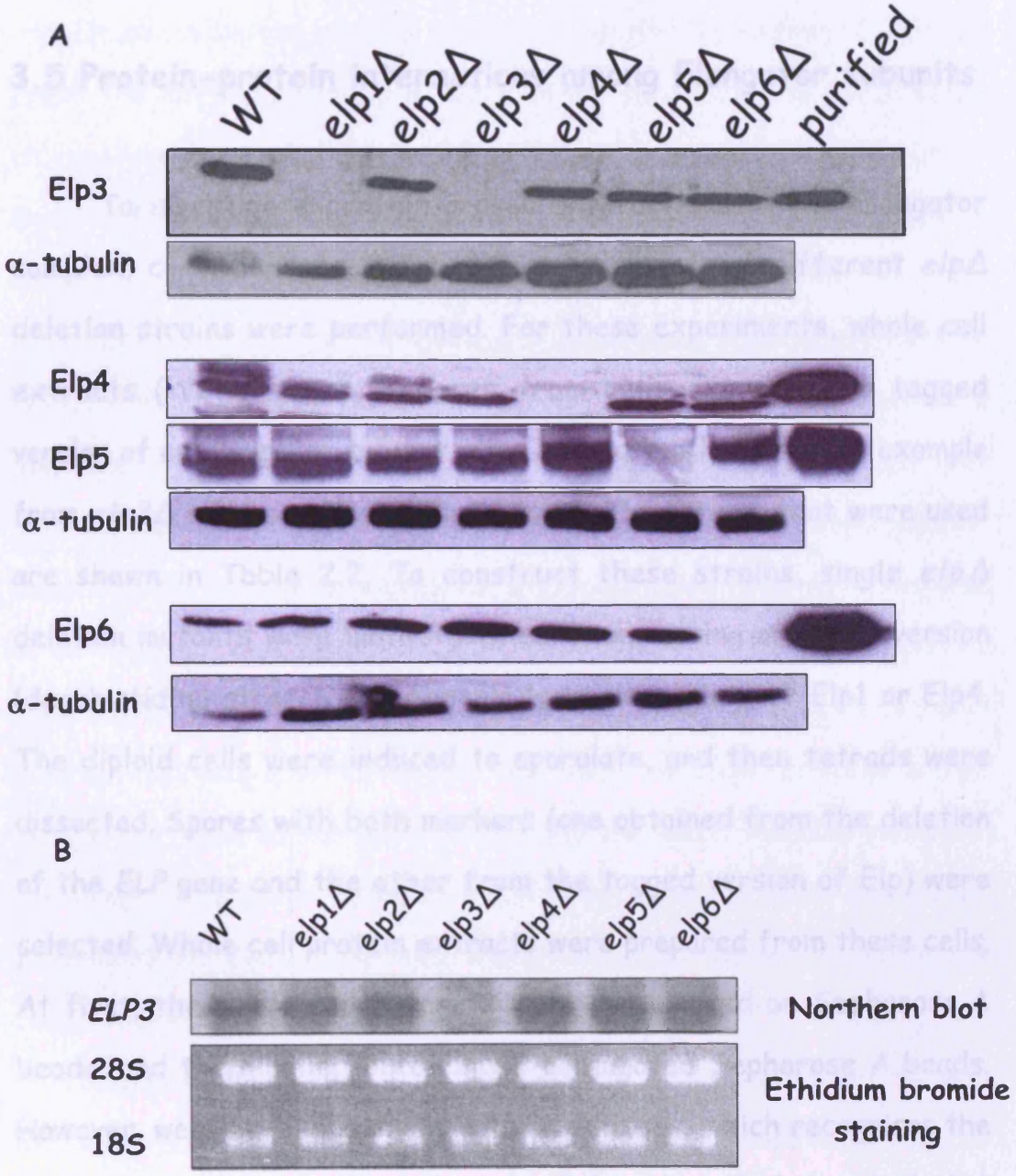

Figure 3.3. Stability and expression of Elongator subunits. ***A***, The presence of Elp3 (upper panels), Elp4 and Elp5 (middle panels), and Elp6 (lower panels) in wild-type and *elpΔ* mutant yeast cells was examined by Western blotting, using anti-Elp antibodies. Detection of α–tubulin was used as a loading control. Note that the relevant Elp4 band in the different cell types is the lower one. The reduced mobility of the Elp4 protein in the control lane with pure Elongator ("purified" lane) is due to the HisHA tag. ***B***, mRNA levels of the *ELP3* gene in the indicated *elpΔ* mutants examined by Northern blotting. Ethidium

bromide staining of 28S and 18S subunits of rRNA was used as a loading control.

3.5 Protein-protein interactions among Elongator subunits

To investigate protein-protein interactions in the Elongator complex, co-immunoprecipitation experiments from different *elpΔ* deletion strains were performed. For these experiments, whole cell extracts (WCEs) were prepared from cells expressing a tagged version of an Elongator subunit while lacking another one, for example from *elp2Δ* cells expressing Elp1-HisHA. The strains that were used are shown in Table 2.2. To construct these strains, single *elpΔ* deletion mutants were mated with cells expressing a tagged version (decahistidine stretch and a single HA epitope tag) of Elp1 or Elp4. The diploid cells were induced to sporulate, and then tetrads were dissected. Spores with both markers (one obtained from the deletion of the *ELP* gene and the other from the tagged version of Elp) were selected. Whole cell protein extracts were prepared from these cells. At first, the whole cell extracts were pre-cleared on Sepharose A beads, and then loaded onto 12CA5-conjugated Sepharose A beads. However, western blot analysis with an antibody, which recognises the HA epitope, showed that only a small fraction of these single-HA tagged proteins (either Elp1 or Elp4) was found on the beads, thereby precluding firm conclusions for the co-immunoprecipitated Elp proteins. It was already known from previous large-scale purification studies that the 12CA5-conjucated Sepharose A beads are able to immunodeplete the single HA-tagged Elp1 protein found in the elution fractions from Bio-Rex 70. Importantly, Elongator was found to elute

as an intact six-subunit complex at moderate salt concentration from this weak cationic resin. Therefore, to enable high-efficiency immunoprecipitation and to increase specificity, extracts derived from the different strains were first subjected to small-scale chromatography on Bio-Rex and the 600 mM potassium acetate elution fractions were then loaded onto 12CA5-conjugated Sepharose A beads.

As shown in Fig. 3.4A, Elongator could under those conditions be specifically immunoprecipitated from wild-type cell extracts expressing tagged Elp1 (right panel), but not from control cells that did not express a tagged Elongator protein (left panel). Remarkably, when the *ELP2* gene was deleted, Elp1 still interacted with the five remaining subunits, although the three smaller proteins Elp4, Elp5 and Elp6 appeared to be somewhat sub-stoichiometric (Fig. 3.4B, left panel). This experiment suggested that the Elp2 protein is not absolutely required for the integrity of the holo-Elongator complex, but may serve as a stabilisation factor between core Elongator, composed of Elp1, Elp2 and Elp3, and the "small" sub-complex composed of Elp4, Elp5 and Elp6.

When extracts from cells lacking the *ELP3* gene were used, only Elp1 was detected in the precipitates, which indicated that none of the remaining five subunits interacts strongly with Elp1 (Fig. 3.4B, middle panel). It should be mentioned that the Elp2 protein levels seemed to be reduced in *elp3Δ* cells, something that was noticed in all the *elpΔ* mutants tested. Unfortunately, as mentioned above the stability of Elp2 in different mutant extracts could not easily be directly assessed by performing western blot analysis on whole cell extracts, because our anti-Elp2 polyclonal antibody was unable to

consistently detect this protein in crude extracts. However, the remaining small amount of Elp2 that could be detected in Bio-Rex eluates was found not to co-immunoprecipitate with Elp1 (Fig. 3.4B, Elp2 blot middle panel). Therefore, because a three-subunit Elp1-Elp2-Elp3 complex does exist as a stable entity (Otero et al., 1999), it can be concluded that Elp1 and Elp2 both interact with Elp3 but not with each other. An additional conclusion is that none of the small subunits interacts strongly with Elp1. Thus Elp3 seems to be essential not only for catalytic function, but also for the integrity of the Elongator complex.

In the absence of Elp4, the Elp1 protein could still interact with Elp3 (Fig. 3.4B, right panel). However, in this case the Elp2, Elp5 and Elp6 proteins could not be detected, even in the inputs. This can be explained in two ways: either these three proteins no longer co-elute with Elp1 and Elp3 from Bio-Rex when Elp4 is absent, or deletion of the *ELP4* gene resulted in reduced Elp2, Elp5 and Elp6 protein levels. However, previous experiments (Fig. 3.3A middle and lower panels) showed that Elp5 and Elp6 protein levels were not decreased significantly in any of the *elpΔ* mutants. Therefore, it is reasonable to conclude that these two proteins do not directly contact Elp1 or Elp3. In the case of the Elp2 protein, firm conclusions on its fate in *elp4Δ* extracts are not possible, because of the poor quality of the anti-Elp2 antibody. However, because previous studies have shown that core Elongator (Elp1/2/3) exists on its own when separated biochemically from the "small" sub-complex (Winkler et al., 2001), the most likely possibility is that the *ELP4* deletion results in reduced Elp2 protein levels. This interpretation would in turn suggest that the presence of Elp4 is not necessary for the integration of Elp2 in core Elongator. In

conclusion, Elp4 is required for stabilisation of Elp2 and the association of the small sub-complex Elp4/5/6 with Elp1.

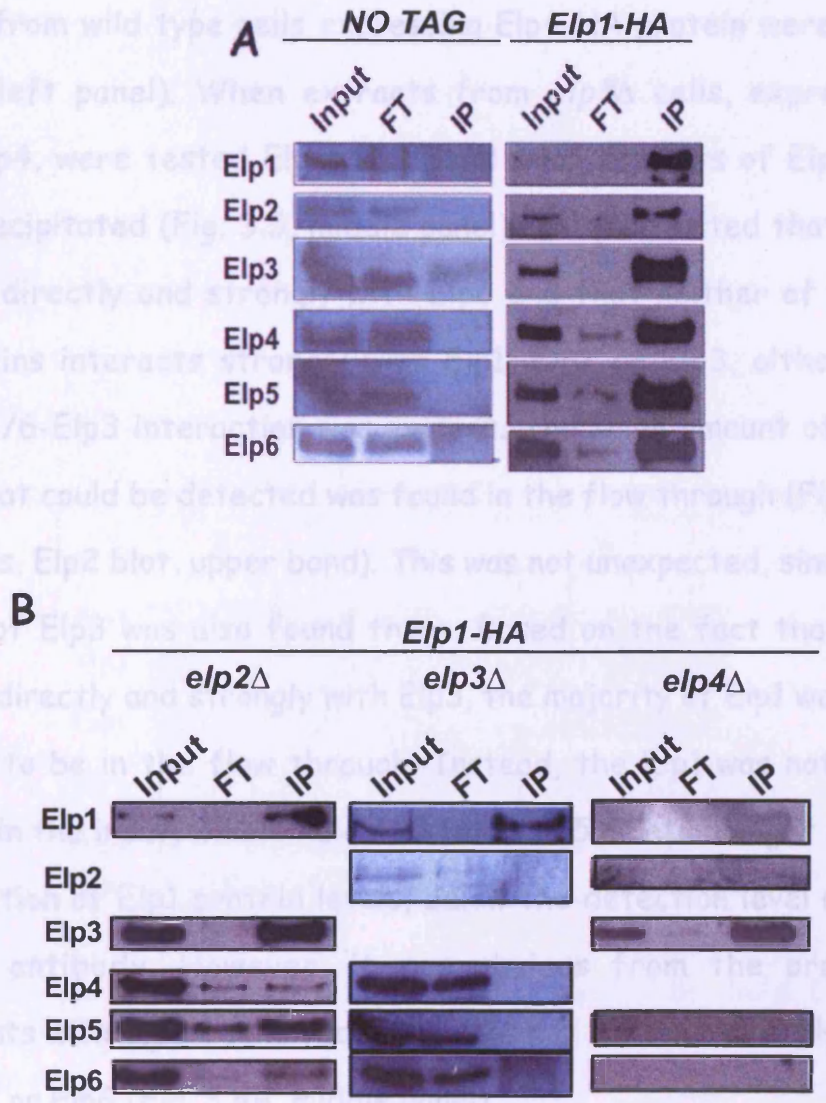

Figure 3.4. *Co*-immunoprecipitation experiments of holo-Elongator subunits *via* Elp1-HA. ***A***, Western blot analysis of the co-immunoprecipitations (co-ips) from cells with untagged or HA-tagged Elp1 protein. The Elp1-HA protein is sufficient to specifically immunoprecipitate the whole Elongator complex (right panel). ***B***, Extracts from the indicated deletion mutants were used for co-ips. The five-subunit complex immunoprecipitated from extracts of *elp2Δ* cells has sub-stoichiometric amounts of Elp4/5/6, suggesting that Elp2 is involved in ensuring the stable interaction of the two sub-

complexes (left panel). Elp1 and Elp2 interact directly with Elp3 but not with each other (middle and right panel). The small sub-complex does not interact directly with Elp1 (middle panel).

Holo-Elongator was also specifically immunoprecipitated when extracts from wild type cells expressing Elp4-HA protein were used (Fig. 3.5 left panel). When extracts from *elp5*Δ cells, expressing tagged Elp4, were tested Elp4, Elp6, and small amounts of Elp3 co-immunoprecipitated (Fig. 3.5, middle panel). This suggested that Elp4 interacts directly and strongly with Elp6 and that neither of these two proteins interacts strongly with Elp1, Elp2 or Elp3, although a weak Elp4/6-Elp3 interaction was evident. The small amount of Elp2 protein that could be detected was found in the flow through (Fig. 3.5, *elp5Δ* cells, Elp2 blot, upper band). This was not unexpected, since the majority of Elp3 was also found there. Based on the fact that Elp1 interacts directly and strongly with Elp3, the majority of Elp1 was also expected to be in the flow through. Instead, the Elp1 was not even detected in the input, which indicated that *ELP5* deletion might result in a reduction of Elp1 protein levels, below the detection level of our anti-Elp1 antibody. However, it was obvious from the previous experiments with *elp3*Δ cells that Elp1 does not interact directly with Elp4, Elp5 or Elp6 (Fig. 3.4B, middle panel).

Finally, when extracts from *elp6*Δ cells carrying a tagged version of Elp4 protein were used, only a small amount of Elp3 was co-precipitated, supporting the notion that these proteins interact, albeit weakly. The absence of a signal for Elp1 and Elp2 can be explained as in the previous cases. However, in this case Elp5 was also not detected, even in the input. Previous experiments showed that the Elp5 protein levels are normal in all *elpΔ* deletion mutants (Fig. 3.3A,

middle panels). This suggested that the Elp5 does not co-elute from the Bio-Rex column with Elp4, which allows the conclusion that Elp5 is incorporated into the small sub-complex in an Elp6-dependent manner.

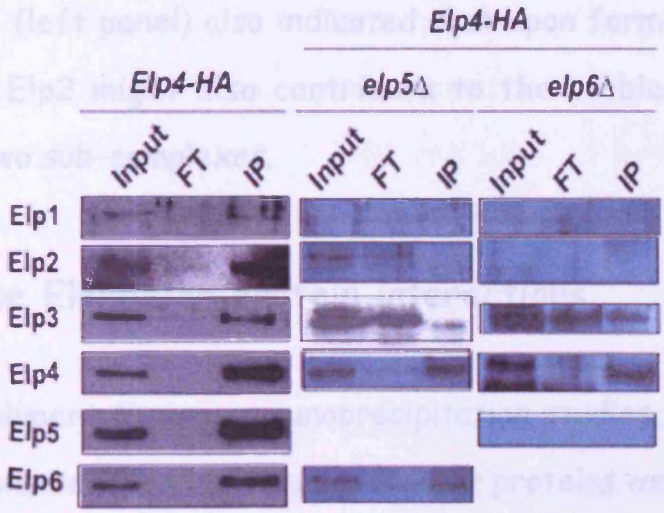

Figure 3.5. Co-immunoprecipitation experiments of holo-Elongator subunits *via* Elp4-HA. HA-tagged Elp4 protein can specifically co-immunoprecipitate the holo-Elongator complex (left panel). Extracts from the indicated deletion mutants were used. Elp4 and Elp5 interact directly with Elp6 but not with each other (middle and right panel). A weak Elp3/Elp4 interaction is also evident (right panel).

The above experiments provided evidence that there is a strong direct interaction between Elp1 and Elp3, as well as between Elp4 and Elp6. They also suggested a direct strong interaction between Elp5 and Elp6. Because core Elongator, consisting of Elp1, Elp2, and Elp3

has previously been isolated (Winkler et al., 2001), the absence of Elp2 in Elp1-precipitates from *elp3Δ* cells also indicated that the interaction with Elp3 is required for the incorporation of Elp2 into core Elongator. Surprisingly, as far as the binary interactions between any individual protein in the Elp4/5/6 module and one of the three larger proteins are concerned, co-immunoprecipitation experiments only indicated a weak interaction between Elp4 and Elp3. This suggests that the formation of the six-subunit Elongator complex is based on the Elp3/Elp4 interaction, which might be strengthened when the two-sub-complexes are incorporated into one complex. However, the result from Fig. 3.4B (left panel) also indicated that upon formation of the holo-complex, Elp2 might also contribute to the stable interaction between the two sub-complexes.

3.6 Pair-wise Elongator protein interactions

To compliment the co-immunoprecipitation studies, *in vivo* pair-wise interactions between individual Elongator proteins were examined by use of the yeast two-hybrid system. The genes encoding all the Elongator subunits were cloned in frame with the activation and the DNA-binding domains of the Gal4 activator, respectively, and a tester yeast strain was transformed with combinations of these 12 plasmids. In this particular strain, expression of the *HIS3* and *ADE2* genes is driven by a promoter, which contains Gal4-responsive elements (the promoter of the *GAL1* and *GAL2* genes in front of the *HIS3* and *ADE2*, respectively). If the two proteins of interest interact directly, the activation domain of Gal4 will be recruited to the promoters of the above genes resulting in their transcriptional activation. This

enables the cells to survive in the absence of histidine and adenine in their growth medium. In order to test whether each Gal4-Elp fusion was functional, the constructed plasmids were also introduced in the corresponding *elpΔ* mutants. For instance, the plasmid encoding an Elp4-Gal4AD fusion protein was introduced in *elp4Δ* cells and the cells were tested for growth at high temperature. In contrast to untransformed mutant cells, transformed cells were insensitive to high temperature, indicating that neither the activation nor the DNA-binding domain of Gal4 interfered with the normal function of the Elongator protein in question.

All the possible combinations of pair-wise Elongator protein interactions were tested in both directions. Surprisingly, the only interaction detected was that of Elp4 and Elp6, in both directions (Fig. 3.6, the many negative results are not shown). Interestingly, this interaction was also observed in the co-immunoprecipitation experiments, further strengthening the conclusion that a strong direct interaction exists between these two proteins. The absence of identification of other positive interactions may point to the limitations of two-hybrid analysis in detecting protein-protein interactions between subunits of multi-subunit complexes, as has also been the case for the yeast TFIIH complex (Feaver et al., 2000).

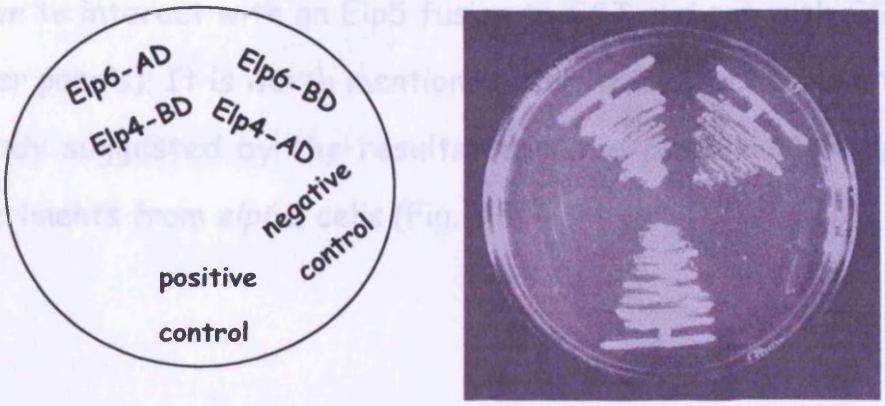

Figure 3.6. Two-hybrid interactions. The reciprocal direct interaction between Elp4 and Elp6 is shown. Yeast cells transformed with the indicated plasmids (left) were grown on medium lacking adenine and histidine (right). The growth of cells is indicative of interaction. Positive and negative controls provided by the manufacturer were used for comparison.

Unfortunately, in this particular case the use of the two-hybrid system thus proved to be unhelpful in identifying the missing pieces in the "interaction map" puzzle. Therefore, I sought to gain evidence for the remaining interactions *in vitro*, by performing pull down experiments, using either recombinant or *in vitro* transcribed-translated Elongator proteins (Fig. 3.7). Recombinant Elp1 and Elp2 proteins tagged with multiple histidines were expressed in insect cells, recombinant GST and GST-Elp5 proteins were expressed in bacteria, and the Elp4 and Elp6 proteins were *in vitro* transcribed-translated. Unfortunately, despite attempts in several expression systems, soluble recombinant, or *in vitro* transcribed-translated, Elp3 protein could not be obtained. The results from binary interaction studies are

shown in Fig. 3.7. Neither Elp1 nor Elp2 was found to interact directly with any of the smaller subunits (upper panels). However, Elp6 was shown to interact with an Elp5 fusion to GST and not with GST alone (lower panels). It is worth mentioning that this direct interaction was already suggested by the results from the co-immunoprecipitation experiments from *elp6Δ* cells (Fig. 3.5, right panel).

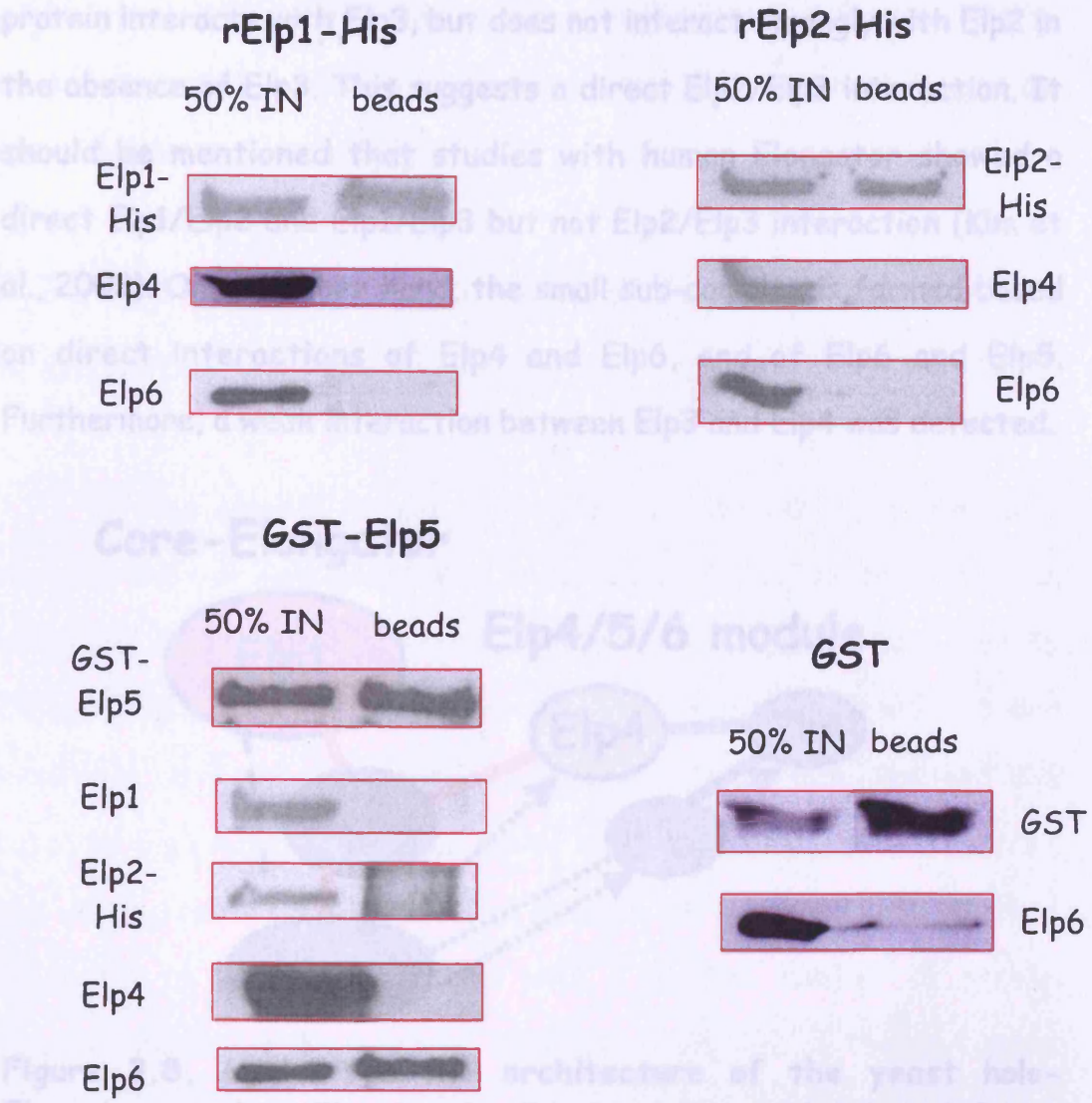

Figure 3.7. *In vitro* pull down experiments. Baculovirus expressed histidine-tagged Elp1 or Elp2 were immobilised on Ni-agarose and incubated with *in vitro* transcribed/translated Elp4 or Elp6 (upper panels). Immobilised GST-Elp5 on GST-Sepharose was incubated with baculovirus expressed Elp1 or Elp2-His, and *in vitro* transcribed/translated Elp4 or Elp6 (lower left panel). *In vitro* transcribed/translated Elp6 was also incubated with immobilised GST (lower right panel). Elp6 interacts directly with GST-Elp5 but not with GST.

Taken together, these results make it possible to propose a model to describe the molecular architecture of the Elongator complex (Fig. 3.8). In the large core Elongator sub-complex, Elp1

protein interacts with Elp3, but does not interact strongly with Elp2 in the absence of Elp3. This suggests a direct Elp2/Elp3 interaction. It should be mentioned that studies with human Elongator showed a direct Elp1/Elp2 and Elp1/Elp3 but not Elp2/Elp3 interaction (Kim et al., 2002). On the other hand, the small sub-complex is formed based on direct interactions of Elp4 and Elp6, and of Elp6 and Elp5. Furthermore, a weak interaction between Elp3 and Elp4 was detected.

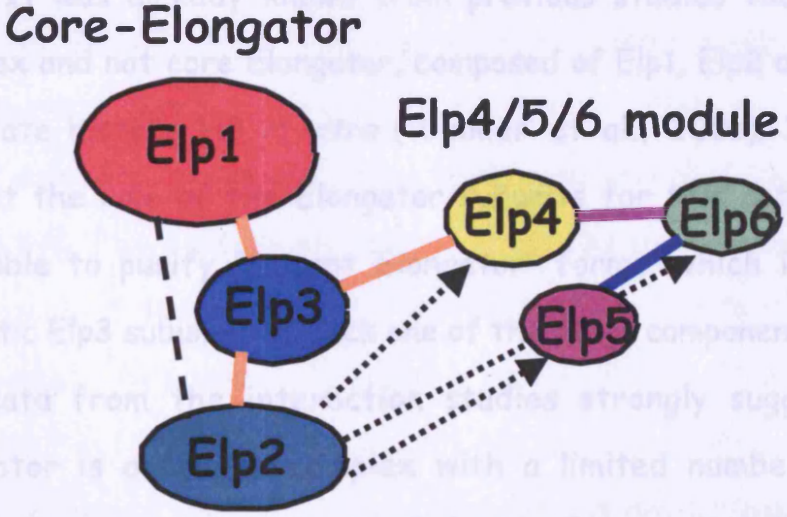

Figure 3.8. Model for the architecture of the yeast holo-Elongator complex. The six subunits are represented with oval shapes and the binary interactions are indicated with lines. Different colors are used depending on the assay that revealed each interaction: orange color for co-immunoprecipitations, blue color for *in vitro* pull down experiments and purple color for an interaction identified by both co-immunoprecipitations and two-hybrid analysis. The dotted arrows are used to denote that Elp2 might interact with any of the Elp4, Elp5 and Elp6 proteins in the context of holo-Elongator. Finally, the dashed line represents the identified interaction between Elp1 and Elp2 in the human Elongator complex.

Moreover, the co-immunoprecipitation studies, suggested that the incorporation of the two sub-complexes into a holo-complex results in a possible interaction of Elp2 with components of the "small" sub-complex and/or the stabilization of the weak Elp3/Elp4 interaction. Uncovered interactions between Elp1 and the "small" sub-complex

might also occur in the context of holo-Elongator complex. Finally, novel interaction surfaces between the two sub-complexes might be formed upon their assembly.

3.7 The WD40 repeat protein Elp2 is dispensable for the *in vitro* HAT activity of Elongator

It was already known from previous studies that the holo-complex and not core Elongator, composed of Elp1, Elp2 and Elp3, can acetylate histone H3 *in vitro* (Winkler et al., 2001). In order to dissect the role of the Elongator subunits for this activity, it was desirable to purify "mutant Elongator" forms, which included the catalytic Elp3 subunit, but lack one of the other components. However, the data from the interaction studies strongly suggested that Elongator is a fragile complex with a limited number of strong interactions among its subunits. Deletion of any one of the *ELP* genes thus more or less completely disrupted the Elongator complex. However, in cells lacking the *ELP2* gene, the remaining five subunits still formed a complex. To investigate if this five-subunit complex retains HAT activity, it was purified and tested in *in vitro* HAT assays. This was particularly relevant because several HAT and HDAC complexes harbor subunits, which – like Elp2 – contain WD40 repeats (Roth and Allis, 1996; Vermaak et al., 1999). It has thus been suggested that WD40 repeats might be involved in contacting histones to facilitate the acetyl-transferase and de-acetylation reactions, respectively.

The purification scheme employed for isolating Elongator from *elp2Δ* cells was identical to that previously used for the purification

of six-subunit Elongator complex (Fig. 3.1A and Winkler et al., 2001). The Western blot analysis of Fig. 3.9A shows that the purified "mutant" complex from *elp2Δ* cells, obtained from two independent preparations, retained the five other Elongator subunits. However, the three smaller subunits as well as the Elp3 protein appeared to be somewhat sub-stoichiometric compared to the wild type complex. In order to be able to compare the HAT activity of the five-subunit complex with that purified from wild type cells, similar amounts of the catalytic subunit Elp3 were used in the HAT assays, with the consequence that other subunits, particularly Elp1, were present in larger amounts in the reactions containing "mutant" *elp2Δ* complex (Fig. 3.9A). Fig. 3.9B shows the result of the HAT assays comparing wild type and the two independently purified "mutant" complexes. Surprisingly, five-subunit Elongator lacking the Elp2 retained full *in vitro* HAT activity directed against histone H3. Adding recombinant, histidine-tagged Elp2 to the reactions did not dramatically affect the activity (the apparent stimulation of wild type complex activity- lane 1 and 2- was due to loading differences). These data demonstrate that the WD40 repeat protein Elp2 is not required for Elongator's HAT activity *in vitro*.

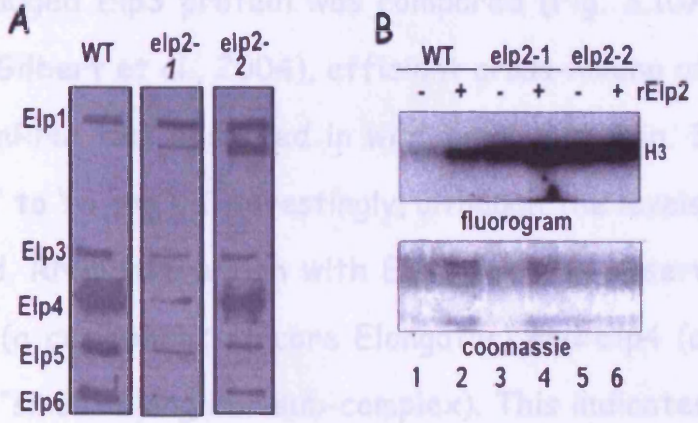

Figure 3.9. The WD40 repeat protein Elp2 is dispensable for the *in vitro* HAT activity of Elongator. ***A,*** Western blot analysis of purified Elongator from wild type and *elp2Δ* cells. Fractions from two independently purified complexes lacking the Elp2 are shown (*elp2-1, elp2-2*). ***B,*** *In vitro* HAT assay using the purified complexes from (*A*). The upper panel is a fluorogram of ^{3}H labelled histone H3 and the lower a coomassie staining of all four core histones used in the assay. Recombinant Elp2 (rElp2), expressed in insect cells, was added in the indicated reactions (2, 4, 6). The amount of rElp2 that was used was comparable with that of yeast Elp2 in the wild type Elongator preparation, as judged by Western blot analysis and silver staining (not shown). Coomassie staining of core histones is shown as a loading control. The mutant form of Elongator derived from *elp2Δ* cells retains full HAT activity.

3.8 Elp3 is important for Elongator-RNA interaction *in vivo*

It was recently shown that the Elongator complex binds to RNA *in vitro* and that it is in close vicinity of nascent pre-mRNA *in vivo* (Gilbert et al., 2004). These results indicated that Elongator is

associated with active genes in living cells. To investigate the subunit requirements for RNA-binding *in vivo*, RNA immunoprecipitation (RIP) experiments were performed and the amount of co-immunoprecipitated *GAL1* mRNA from wild type, *elp2Δ* and *elp4Δ* cells expressing Myc-tagged Elp3 protein was compared (Fig. 3.10A). As shown previously (Gilbert et al., 2004), efficient cross-linking of Elp3 protein to *GAL1* mRNA was observed in wild type cells (Fig. 3.10A, compare 'Elp3-myc' to 'no tag'). Interestingly, although the levels were somewhat reduced, RNA interaction with Elp3 was also observed in cells lacking Elp2 (a component of core Elongator) and Elp4 (a core component of the "small" Elongator sub-complex). This indicates that neither the "small" sub-complex, nor Elp2 play crucial roles in the association of Elongator (Elp3) with transcripts *in vivo*. Because Elp3 levels are dramatically reduced in cells lacking Elp1 (Fig. 3.3A upper panels), it was not relevant to compare the immunoprecipitated RNA from wild type and *elp1Δ* cells. However, by tagging Elp1 rather than Elp3, the importance of the Elp3 protein for Elongator-RNA interaction was investigated (Fig. 3.10B). Remarkably, Elp1-RNA interaction was dramatically reduced in the absence of Elp3 (Fig 3.10B, upper panel), and this decrease was not due to changes in the expression level of the Elp1 protein (Fig. 3.10B, lower panel). Taken together, these data indicate that the Elp3 protein plays a crucial role for the association of Elongator with RNA from active genes.

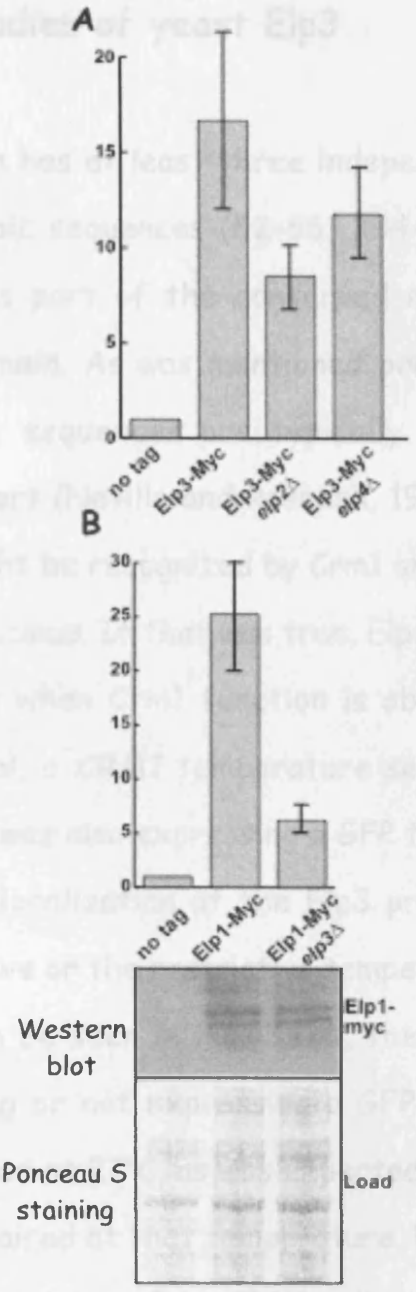

Figure 3.10. Elp3, but not Elp2 and Elp4, is required for Elongator-RNA association *in vivo*. RNA-immunoprecipitation experiments using extracts from strains expressing 18myc-tagged Elongator subunits. RNA was quantitated using reverse transcriptase real-time PCR. Bars represent the average of two independent experiments. Error bars indicate variance. **A**, RIP experiments with the indicated strains expressing myc-tagged Elp3 or the untagged control. **B**, RIP experiments with the indicated strains expressing myc-tagged Elp1 or the untagged control (upper panel). Lower panel shows the result of an Elp1-myc Western blot with loading control (Ponceau S-stained membrane section) to demonstrate that Elp1 is stable in *elp3Δ* cells. The resolution of Elp1 into two distinct bands has previously been described (Fichtner et al., 2003).

3.9 Localization studies of yeast Elp3

The Elp3 protein has at least three independent stretches of leucine-rich hydrophobic sequences (52-66, 354-365, 454-462 aa). The latter of these is part of the conserved motif D of histone acetyl-transferase domain. As was mentioned previously (paragraph 3.1) such hydrophobic sequences are typically critical for Crm1-dependent nuclear export (Neville and Rosbash, 1999). Therefore, we reasoned that Elp3 might be recognized by Crm1 and be shuttled from the cytoplasm to the nucleus. If that was true, Elp3 should accumulate in the nucleus of cells when Crm1 function is abrogated. Since the *CRM1* gene is essential, a *CRM1* temperature sensitive (ts) mutant strain was used, which was also expressing a GFP tagged Elp3 protein. Using this strain, the localization of the Elp3 protein in living cells growing at the permissive or the restrictive temperature was followed microscopically. As can be seen in Fig. 3.11A, these ts mutant *CRM1* cells, either expressing or not expressing a GFP-tagged Elp3, grew normally at 30°C but died at 37°C, as was expected since the essential function of Crm1 is impaired at that temperature. Fig. 3.11B shows the GFP and DNA (DAPI) staining of ts *CRM1* cells at 30°C (permissive) and 37°C (restrictive temperature) expressing (lower panels) or not expressing (upper panels) the GFP-Elp3 protein. The vast majority of Elp3-GFP cells showed cytoplasmic GFP staining at both temperatures. A few cells showed co-localization of GFP and DNA at 37°C, suggesting an accumulation of Elp3 in the nucleus (Fig. 3.11C). However, these cells did not exceed the 5% of the total cell population, which cannot be considered significant.

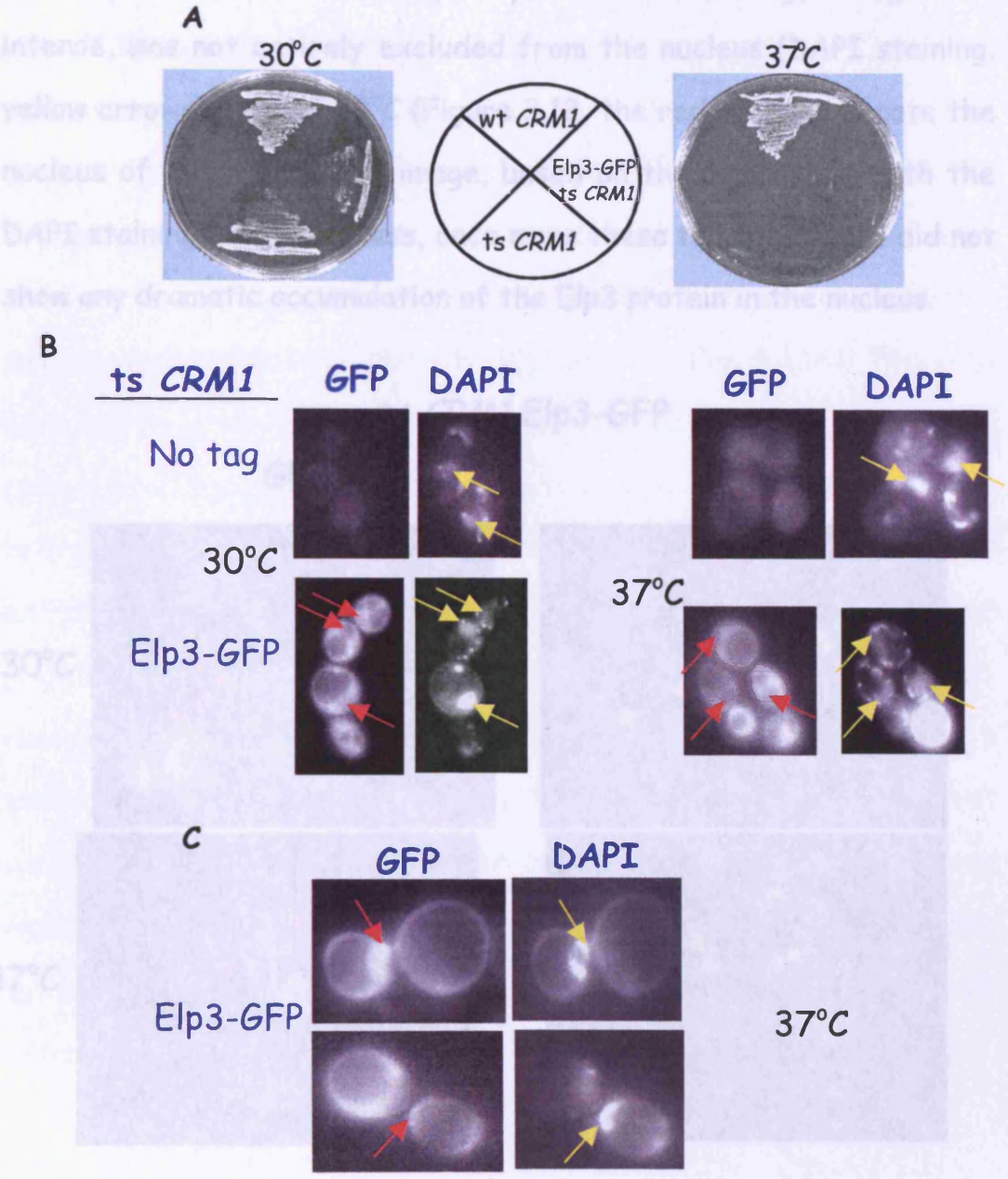

Figure 3.11. Elp3 is mainly cytoplasmic in *CRM1 ts* mutant cells. ***A,*** Comparison of growth of wild type yeast cells with those carrying or not a GFP-tagged Elp3 and a temperature sensitive *CRM1* gene. *CRM1* ts cells do not grow at 37°C. ***B,*** GFP staining of untagged or Elp3-GFP tagged ts *CRM1* cells. The majority of cells show cytoplasmic staining at both 30°C and 37°C. Note the apparently empty space (the nucleus) that is marked by red arrows on a GFP image. Yellow arrows indicate the DNA staining. ***C,*** In a few cells the Elp3-GFP staining (red arrows) co-localizes with DNA (DAPI staining, yellow arrows), suggesting an accumulation of Elp3 in the nucleus.

Additionally, when the ts *CRM1* Elp3-GFP cells were first fixed and then examined microscopically, the GFP staining, though less intense, was not entirely excluded from the nucleus (DAPI staining, yellow arrows) even at 30°C (Figure 3.12, the red arrows indicate the nucleus of a cell on a GFP image, based on the comparison with the DAPI staining). Nevertheless, once more these ts mutant cells did not show any dramatic accumulation of the Elp3 protein in the nucleus.

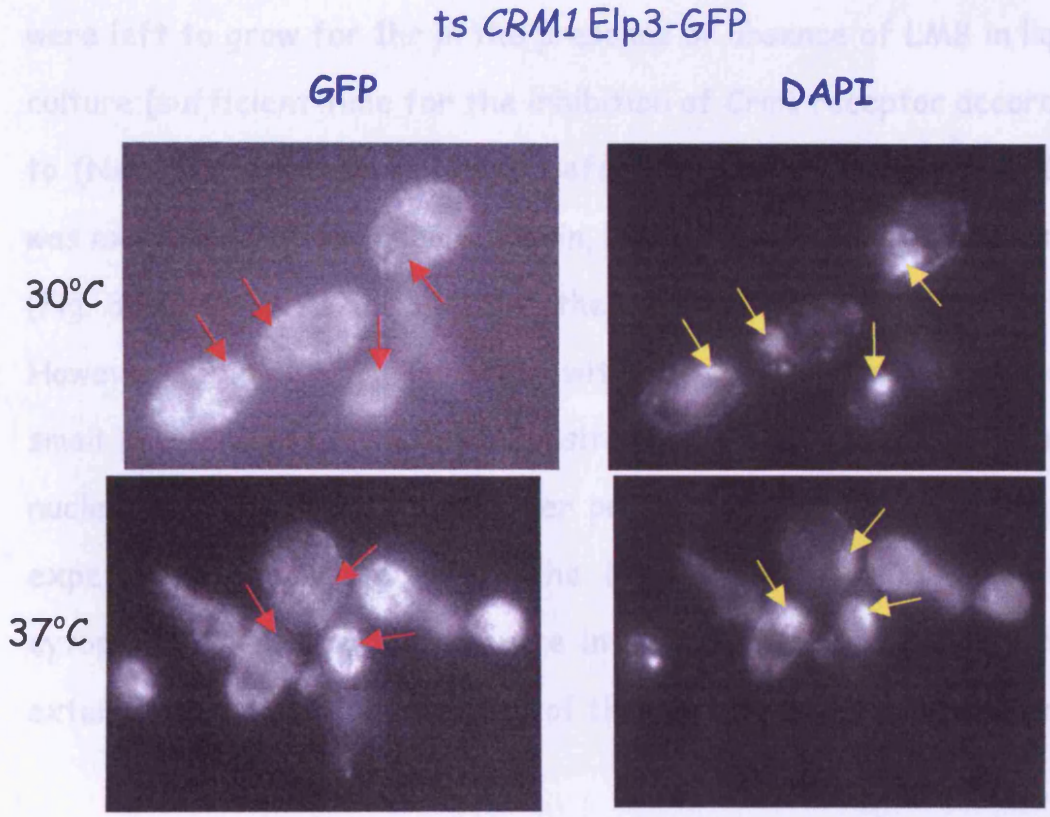

Figure 3.12. GFP and DAPI staining of fixed ts *CRM1* cells expressing an Elp3-GFP fusion. Temperature sensitive *CRM1* cells expressing or not expressing the Elp3-GFP fusion protein were grown to mid-logarithmic phase at 30°C. Then, they were split and half of the population was fixed with methanol directly, whereas the other half was transferred for 1 hr at 37°C before fixing. After fixing, cells were stained with DAPI and studied microscopically for GFP and DNA staining. The yellow arrows mark the DAPI-stained DNA (nucleus), whereas the red arrows mark the nucleus of the same cell on the corresponding GFP image.

Next, another mutant strain for the Crm1 protein was used, which expresses a Crm1 protein with a single point mutation (*CRM1*T539C). This *crm1* alleles confer sensitivity to the *Streptomyces* metabolite Leptomycin B (LMB) (Neville and Rosbash, 1999). A strain, which carries the Elp3-GFP fusion in the *CRM1*T539C background was constructed (the original strain was a kind gift from Dr. John F. Diffley). As expected, these cells could not grow on rich medium supplemented with LMB (100 ngr/ml) (Fig. 3.13A). The cells were left to grow for 1hr in the presence or absence of LMB in liquid culture (sufficient time for the inhibition of Crm1 receptor according to (Neville and Rosbash, 1999)) before the localization of Elp3-GFP was examined microscopically. Again, Elp3-GFP was mainly cytoplasmic (Fig. 3.13B compare "no tag" and the upper panel of Elp3-GFP cells). However, as in the previous case with the ts *CRM1* mutant strain, a small percentage of the LMB sensitive cells also showed prominent nuclear staining (middle and lower panels of Elp3-GFP cells). These experiments suggested that the Elp3 protein, which is mainly cytoplasmic, does not accumulate in the nucleus to any significant extend as a result of inactivation of the Crm1 import/export factor.

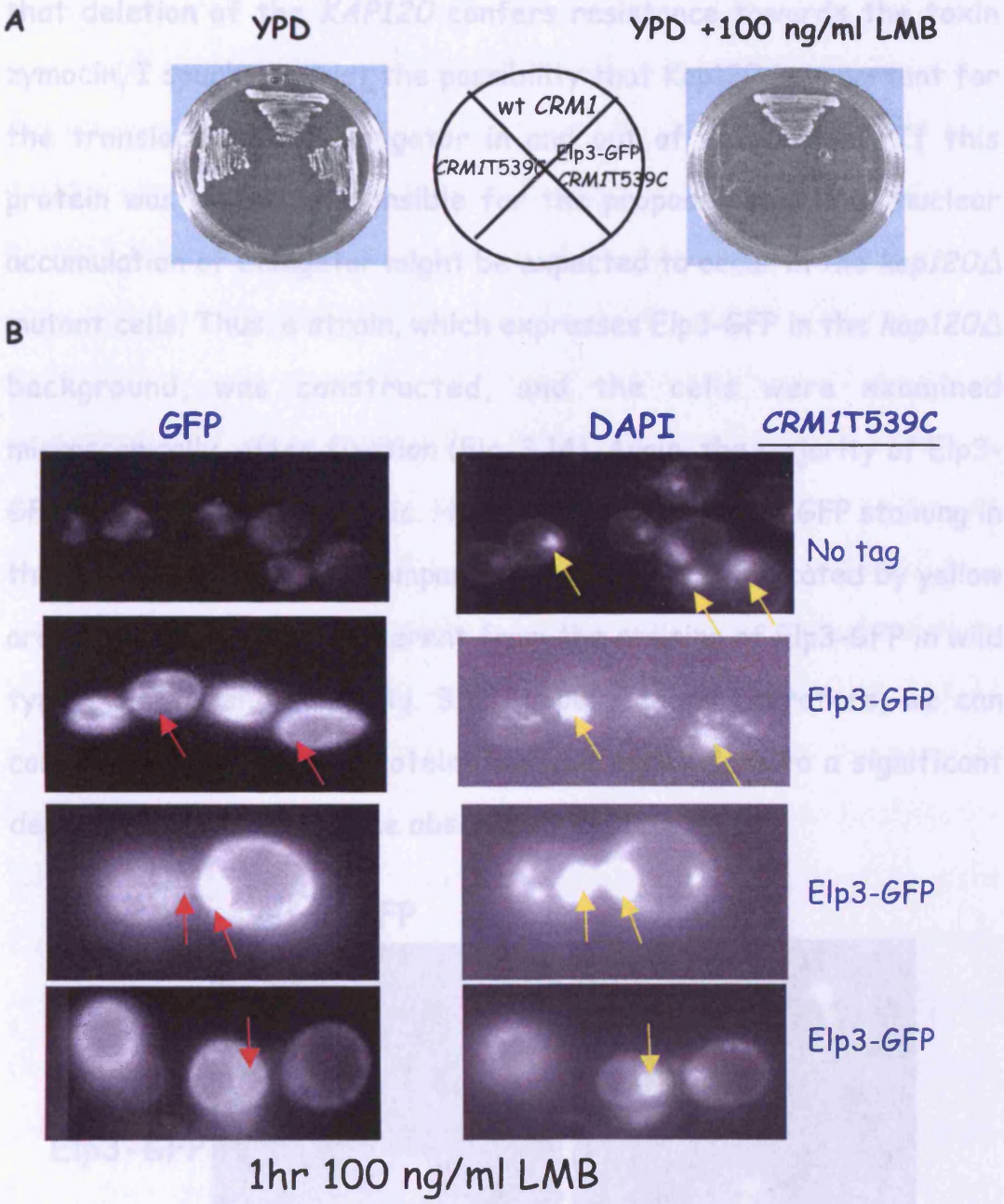

Figure 3.13. LMB-dependent inhibition of Crm1 function is not sufficient for Elp3 accumulation in the nucleus. *A*, Growth of wild type, *CRM1*T539C untagged, and *CRM1*T539C Elp3-GFP tagged mutant cells at 30°C on a rich medium, in the absence or presence of LMB. *B*, GFP and DAPI (yellow arrows) staining of untagged or Elp3-GFP tagged living cells after incubation with LMB for 1hr. The red arrows mark the corresponding position of the nucleus on a GFP image, based on the comparison with the DAPI staining.

Finally, based on the observation from Fichtner et al. (2003), that deletion of the *KAP120* confers resistance towards the toxin zymocin, I sought to test the possibility that Kap120 is important for the translocation of Elongator in and out of the nucleus. If this protein was indeed responsible for the proposed shuttling, nuclear accumulation of Elongator might be expected to occur in the *kap120Δ* mutant cells. Thus, a strain, which expresses Elp3-GFP in the *kap120Δ* background, was constructed, and the cells were examined microscopically, after fixation (Fig. 3.14). Again, the majority of Elp3-GFP staining was cytoplasmic. However, there was weak GFP staining in the nucleus (red arrows, compare to DAPI staining indicated by yellow arrows), but not at all different from the staining of Elp3-GFP in wild type cells, as shown in Fig. 3.12 (upper panel). Therefore, we can conclude that the Elp3 protein does not accumulate to a significant degree in the nucleus in the absence of Kap120.

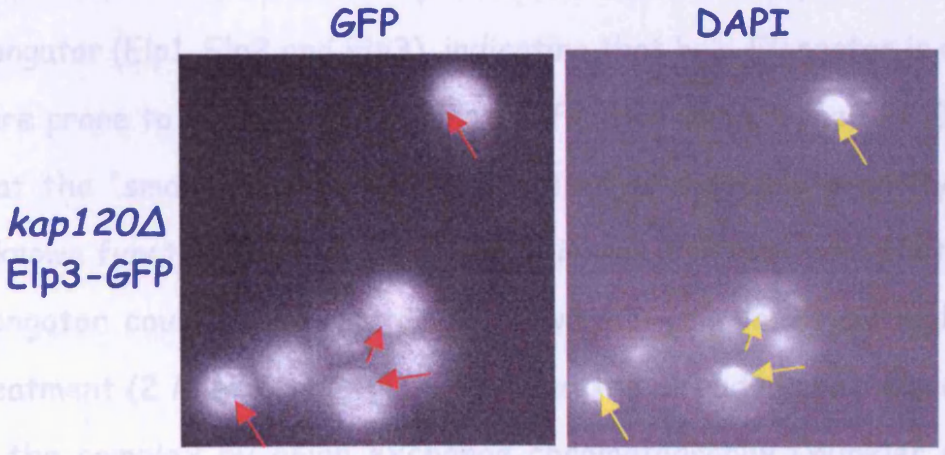

Figure 3.14. GFP and DAPI staining of fixed *kap120Δ* Elp3-GFP cells. *kap120Δ* mutant cells expressing the Elp3-GFP fusion protein were grown to mid-logarithmic phase at 30°C and then fixed with methanol, stained with DAPI and studied microscopically for GFP and DNA staining. The yellow and red arrows are used as previously.

3.10 Discussion and conclusions

The experiments described in this Chapter represent the first comprehensive investigation of the structure-function relationship of yeast Elongator, a histone acetyl-transferase complex with a role in RNAPII transcription. The existence of six-subunit Elongator complex was confirmed biochemically and genetically and its detailed molecular architecture was revealed. Moreover, several lines of evidence suggested roles, or unexpected lack of roles, for individual Elongator subunits (Petrakis et al., 2004). Finally, the idea that Elongator shuttles continuously from the cytoplasm to the nucleus was tested.

Six-subunit holo-Elongator complex could be isolated following the purification of a tagged version of the Elp4 protein (Fig. 3.1). It should be noted that no other polypeptides were found to co-purify with Elongator under these conditions. However, upon purification the "small" sub-complex was super-stoichoimetric compared to core Elongator (Elp1, Elp2 and Elp3), indicating that holo-Elongator is either more prone to disintegrate during purification when tagged on Elp4 or that the "small" sub-complex also exists as a separate entity with unknown function. During purification, it was also observed that holo-Elongator could be separated into two sub-complexes by high salt treatment (2 M NaCl, Fig. 3.1). This and the already known disruption of the complex by anion exchange chromatography (Winkler et al., 2001), makes it reasonable to conclude that holo-Elongator is a fragile complex.

Genetic analysis using cells lacking any one of the six *ELP* genes or even a combination of two showed that these cells display the same phenotypes (Fig. 3.2). These include slow growth in the presence of high salt and sensitivity to high temperature, suggesting participation

of all the Elp proteins in the same cellular process. Moreover, Western blot analysis, where the protein levels of the Elongator subunits were detected in wild type and all the *elpΔ* mutant cells, revealed that the Elp3 protein is susceptible to degradation in the absence of the other holo-Elongator components, but in particular in the absence of Elp1 (Fig.3.3). Structural interdependence among the components of multi-subunit complexes is a common feature of such complexes, and this finding also supports the idea that the six Elongator proteins form a complex in yeast cells.

One interesting issue that remains to be re-addressed is the composition of Elongator complex isolated from the chromatin salt-stable fractions of yeast whole cell extracts. Based on the available data, it seems likely that all the six subunits would be detected. However, an interesting speculation is that the "small" sub-complex functions as a "loading" factor of core Elongator on ternary complexes, and is therefore associated only with the soluble, DNA-free fraction of the complex.

Work by Schaffrath and co-workers using immunoprecipitation of tagged Elongator subunits and detection of a co-precipitated differently tagged subunit gave some information about subunit requirements for Elongator assembly (Fichtner et al., 2002; Frohloff et al., 2003). While the present work confirms and significantly extends the conclusions derived from these studies, there are also surprising contradictions. For example, Frohloff et al. (Frohloff et al., 2003) detected the Elp3 protein in cells lacking *ELP1*, whereas the above results indicated that normal Elp3 stability *in vivo* requires *ELP1* (Fig. 3.3A). Likewise, these authors concluded that the structural integrity of the small Elp4/5/6 sub-complex requires the *ELP4*, *ELP5*,

and *ELP6* genes, while this work showed that Elp4 can interact with Elp6 in the absence of *ELP5* (Fig. 3.5, middle panel). The reasons for these differences are presently unclear.

Three different approaches were followed in order to identify interactions between the six Elongator subunits. The results are summarized in the proposed model of Fig. 3.8. Briefly, core Elongator is formed as a result of a direct interaction of Elp1 and Elp2 with Elp3. A direct strong interaction of Elp6 with Elp4 and Elp5 is sufficient for the assembly of the "small" sub-complex. The only detected interaction between subunits of the two sub-complexes, albeit weak, was between Elp3 and Elp4. We therefore favor the idea that interactions between subunits of the two sub-complexes primarily take place in the context of holo-Elongator. In support of this hypothesis the absence of Elp2 was shown to de-stabilize the interaction of the "small" sub-complex with Elp1 and Elp3 (Fig. 3.4), though no direct binary interaction was detected between Elp2 and any of the Elp4/5/6 proteins.

It was shown previously for other multi-protein complexes that different subunits have distinct roles (Lewis and Reinberg, 2003; Sterner and Berger, 2000; Sudarsanam and Winston, 2000). It was, therefore, interesting to dissect the requirement of yeast Elongator subunits for the two identified functions of the complex, the *in vitro* HAT activity and the *in vivo* RNA-binding. In the case of the *in vitro* HAT activity, "mutant" complexes, which would include Elp3 but lack one or more of the other subunits, had to be purified to homogeneity and their activity compared with that of wild type six-subunit Elongator. However, in order for these studies to be relevant and possible, stable mutant Elongator forms had to exist in different *elpΔ*

mutant cells. Unfortunately, the co-immunoprecipitation experiments described in Fig. 3.4 and 3.5, clearly showed that Elongator is a fragile complex, which typically dissociated when just one of its subunit proteins was absent. For the *in vivo* RNA-binding, the Elongator-RNA interaction in wild type and different *elpΔ* mutants was compared.

One of the hypothesis for the architecture of the Elongator complex was that the Elp2, a WD40 repeat-containing protein, would turn out to either be essential for the integrity of the Elongator complex, or be required for its HAT and RNA-binding activity. Surprisingly, in the *elp2Δ* mutant cells the remaining five Elp proteins were shown to still form a complex, indicating that the Elp2 protein is not crucial for the integrity of Elongator. It should be mentioned that data from Schaffrath and co-workers suggested that the role of the Elp2 protein might be to allow interactions with other proteins, such as Kti12 (Fichtner et al., 2002).

Next, the "mutant" form of Elongator from *elp2Δ* cells was purified and tested for its ability to acetylate core histones *in vitro*. Fig. 3.9 shows that this complex retained the ability to acetylate histone H3, at levels comparable to that of wild type Elongator. This result indicated that the WD40 repeats of Elp2 are not crucial for the contacts between the complex and core histones that are presumably required to direct the catalytic activity of Elp3 to its substrate. Considering that the stability of Elp3 is Elp1-dependent (Fig. 3.3), this indicated that one, two or all the "small" subunits (Elp4/5/6) are necessary to form an active HAT with the dimer of Elp1/Elp3.

In contrast to the Elp2 protein, the Elp3 subunit appeared to play a crucial role for the integrity of holo-Elongator complex (Fig.

3.4B). When it was missing, the "small" sub-complex did not associate with the Elp1 protein. This is consistent with the finding that among the subunits of the two sub-complexes, only the Elp3 was found to interact weakly with Elp4 (Fig. 3.5).

Recently, Elongator was shown to interact co-transcriptionally with mRNA (Gilbert et al., 2004). Using extracts from different *elpΔ* mutants it was clear that the minimal "Elongator complex" capable of interacting with mRNA is the Elp1/Elp3 dimer (Fig. 3.10). In light of the fact that Elp3, but not the other subunits, is conserved from archeae to man (Wittschieben et al., 1999), it was perhaps not surprising to find that this subunit is also crucial for the *in vivo* RNA binding of Elongator. It thus seems reasonable to expect that the fundamental functions of the Elongator complex be supplied by abilities intrinsic to Elp3, with the other subunits playing primarily function-augmentative or regulatory roles. It would be very interesting to know whether the HAT domain, located at the C-terminus (Wittschieben et al., 1999), or the SAM-binding domain, recently identified at the N-terminus of Elp3 (Chinenov, 2002), has any role in RNA binding of Elongator. Moreover, it would be interesting to test whether Elongator, though not essential for the cell, has an additional auxiliary role for the stability of RNA during transcription, its accurate splicing, or even its translocation and further processing in the cytoplasm.

During the last few years, one controversial issue concerning Elongator is its sub-cellular localization. Genetic and biochemical data clearly suggested a role for Elongator in the nucleus (Gilbert et al., 2004; Otero et al., 1999; Wittschieben et al., 2000; Wittschieben et al., 1999). However, two immune-localization studies failed to detect

significant amounts of the yeast complex in the nucleus (Fichtner et al., 2002; Pokholok et al., 2002), whereas, Fichtner et al. (Fichtner et al., 2003) recently showed that the Elp1 protein has an intrinsic nuclear localization signal, sufficient to direct the green fluorescent protein (GFP) from the cytoplasm to nucleus. Moreover, in the latter study (Fichtner et al., 2003), Schaffrath and co-workers suggested a model according to which nuclear retention of Elongator was mediated by a member of the importin β family (Kap120), which might be crucial for the mechanism of action of the toxin zymocin (for details see at paragraph 1.3 of Chapter 1). Triggered by this suggestion I sought to study whether Elongator shuttles actively from nucleus to cytoplasm *via* the action of an import-export receptor.

The best-studied receptor, the function of which has been linked to translocation of a number of different factors in *S. cerevisiae* is the Crm1 protein (Neville and Rosbash, 1999). Therefore, if Crm1 mediates Elongator shuttling, Elp3 should accumulate in the nucleus when the function of Crm1 is impaired. Using different *crm1* mutant strains, the localization of an Elp3-GFP fusion protein was studied under the microscope. Under all conditions tested, the great majority of detectable Elp3 was found in the cytoplasm. Only a few cells showed a clear nuclear signal. However, these did not represent a significant proportion of the whole population. Moreover, deletion of *KAP120* did not result in any accumulation of Elp3 in the nucleus, in contrast to what was speculated by Fichtner et al. (Fichtner et al., 2003). It should be mentioned that especially in the case of fixed cells, the Elp3-GFP signal was also evident in the nuclear region (according to a comparison with DAPI staining), though it was less intense than the cytoplasmic signal. This suggests the presence of a

low amount of Elongator in the nucleus, which might be sufficient for its transcription-related functions. Nevertheless, the mechanisms governing Elongator localization in cells remain unclear. Therefore, it would be interesting for this study to be extended to investigating the role of different growth conditions, such as growth in the presence of the toxin zymocin, or under stress as well as the role of other import-export receptors in the cellular localization of Elongator complex. Finally, it would be of great interest to uncover Elongator's possible cytoplasmic role (see also discussion in Chapter 5).

CHAPTER 4

KTI12 AND ELONGATOR: IN VITRO AND IN VIVO STUDIES OF THEIR RELATIONSHIP AND IMPLICATIONS FOR ZYMOCIN ACTION

4.1 Introduction

As mentioned in the Introduction (Chapter 1), the *KTI12* and *ELP* genes were identified in a genetic screen for targets of the toxin zymocin in *S. cerevisiae* cells (Frohloff et al., 2001). Studies from Schaffrath and co-workers suggested a physical interaction between Elongator and Kti12 (Fichtner et al., 2002a). In this Chapter, the role of Kti12 in the *in vitro* and *in vivo* function of Elongator is explored. Moreover, genetic and biochemical experiments, which test the possibility that the mechanism of zymocin action involves RNAPII degradation, are presented.

4.2 Physical association of Elongator and Kti12

While Kti12 was not found to associate with purified holo-Elongator (Winkler et al., 2001), Fichtner et al. have reported a physical interaction using immunoprecipitation assays at very low stringency (Fichtner et al., 2002a). In order to test the stability of the Kti12-Elongator interaction, co-immunoprecipitation experiments were performed using extracts from cells expressing a 6-HA affinity tagged Kti12 protein. Fig. 4.1 shows the western blot analysis of immunoprecipitations (IPs) with an anti-HA antibody using extracts from cells expressing untagged or tagged Kti12. In the control IP from untagged cells, none of the Elongator proteins were immunoprecipitated (lane 3), while 6-HA tagged Kti12 co-immunprecipitated significant amounts of Elp3 and Elp4 under low salt conditions (250 mM, lane 6). By contrast, under more stringent conditions (500 mM, lane 8), there was significantly less Elongator

associated with Kti12. However, both Elp3 and Kti12 were clearly co-depleted from the flow through also under these conditions (compare lane 4 and 7 of upper and middle panel), suggesting that Elp3 is washed off the beads by the 500 mM salt wash. Taken together, these data indicate that Elongator and Kti12 interact in a salt-labile manner.

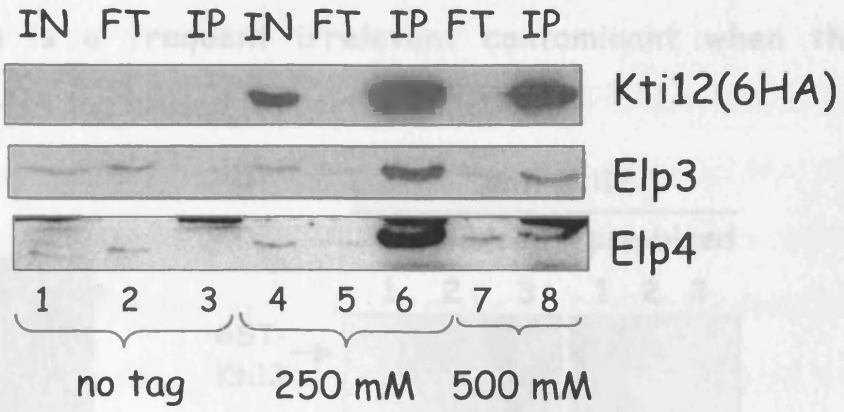

Figure 4.1. Co-immunoprecipitation of Elongator and Kti12. Extracts from cells expressing untagged or 6xHA tagged Kti12 were used for immunoprecipitation experiments, using an anti-HA antibody. Under low stringency conditions (250 mM salt), immunoprecipitated Kti12(6HA) co-precipitate significant amounts of Elp3 and Elp4 (lane 6). When higher salt concentration was used, significantly less Elongator was found in the precipitate (lane 8).

4.3 Purification of Kti12 from soluble fraction of yeast whole cell extracts

Since Kti12 was found to interact with Elongator, it was interesting to test the possibility that these proteins might exist in the same complex. The genomic *KTI12* gene was, therefore, modified so that the Kti12 protein was expressed with a C-terminal decahistidine-HA (single) tag. After genetic confirmation that the epitope tag did not interfere with Kti12 function, the modified Kti12

protein was purified from the soluble fraction of yeast whole cell extracts, by a mixture of conventional and affinity chromatography, following the same scheme that was used previously for holo-Elongator (Fig. 3.1 and Winkler et al., 2001). Fig. 4.2A shows a coomassie-stained gel of highly purified Kti12 obtained from that purification. The ~43 kDa protein was identified by mass spectrometry and found to be Kti12. The 180 kDa was identified as the product of *YDL223C* gene. This protein is a frequent irrelevant contaminant when the decahistidine-HA tag is used (Gilbert et al., 2003).

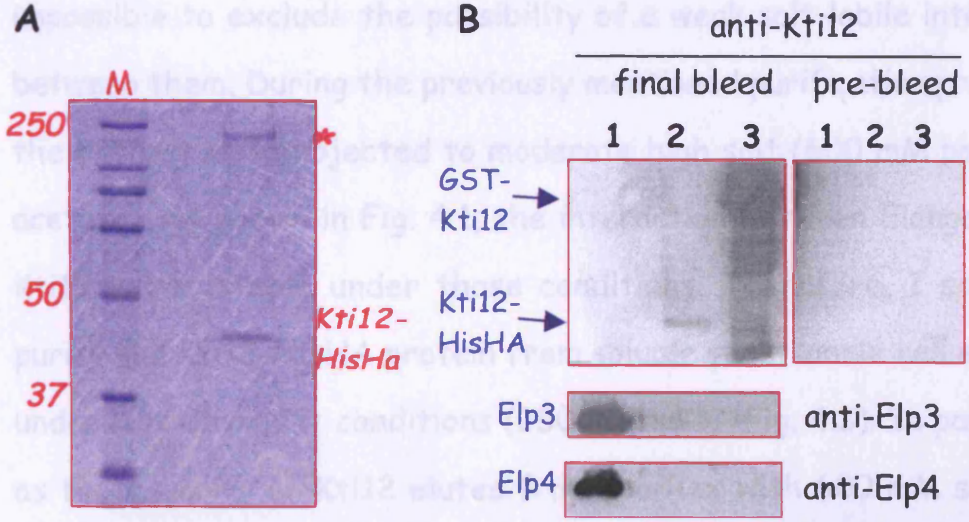

Figure 4.2. Purification of soluble Kti12 from yeast whole cell extracts. *A*, Coomassie stained gel of highly purified Kti12. The protein indicated with asterisk is the irrelevant contaminant, encoded by the *YDL223C* gene. M indicates marker proteins. *B*, Western blot analysis using anti-Kti12, anti-Elp3 and anti-Elp4 antibodies. The final anti-Kti12 bleed, but not the pre-bleed, recognises the recombinant GST-Kti12 protein (lane 3, left upper panel) and the purified yeast Kti12-HisHA (lane 2, left upper panel). Highly purified native yeast Kti12 does not contain Elp3, or Elp4 (lane 2, lower panels), and highly purified Elongator does not contain Kti12 (lane 1, left upper panel).

Max Soegaard, another PhD student in the lab, raised a rabbit antibody against a GST-Kti12 fusion protein (see Chapter 2). The final bleed but not the pre-bleed, recognised the highly purified Kti12-

HisHA protein from yeast (lane 2) as well as the recombinant GST-Kti12 protein (lane 3) (Fig. 4.2B left upper panel). Using this antibody, it was also shown that highly purified holo-Elongator complex does not contain even small amounts of Kti12 (Fig. 4.2B left upper panel, lane 1). Conversely, highly purified yeast Kti12 (lane 2 of left upper and lower panels) did not contain even small amounts of either Elp3 or Elp4 (Fig. 4.2B lower panels, compare lane 1 where holo-Elongator is loaded with lane 2).

The method used to purify holo-Elongator and Kti12 made it impossible to exclude the possibility of a weak salt-labile interaction between them. During the previously mentioned purification procedure the extract was subjected to moderate high salt (600 mM potassium acetate). As shown in Fig. 4.1, the interaction between Elongator and Kti12 is not stable under those conditions. Therefore, I sought to purify the Kti12-HisHA protein from soluble yeast whole cell extracts under less stringent conditions (250 mM salt) (Fig. 4.3). In particular, as the majority of Kti12 elutes from Bio-Rex with 600 mM salt, this eluate was split into two, half of which was further processed at 600 mM salt, whereas the other was diluted to 250 mM prior to loading on 12CA5-conjugated Sepharose A beads. The elution profile after the third step of the purification scheme (Ni-agarose) was followed by Western blot analysis. Kti12 and Elp3 were found to co-elute only under low salt conditions (Fig. 4.3A, elution from Ni-agarose with increasing amounts of imidazole). However, in order to investigate whether Elp3 and Kti12 were also in the same complex, the proteins in the 500 mM imidazole elution fraction were subjected to gel filtration chromatography. Fig. 4.3B shows a Western blot analysis of Elp3 and Kti12-HA from the different elution fractions of the sizing column.

Although the resolution of this gel filtration experiment is not very good, two observations can be made: (i) There is a good but not precise overlap of the elution profile for Elp3 and Kti12 (Elongator peaks in lane 5, whereas Kti12 in lane 6) and (ii) "Elongator-free" Kti12 (lane 7) elutes as a protein of higher molecular weight than predicted from its sequence (37 kDa). The fraction where Elongator peaks (lane 5) also contains significant amounts of Kti12, suggesting that under low stringency conditions a fraction of Kti12 associates with Elongator. Finally, the elution of "Elongator-free" Kti12 at molecular size >37 kDa suggested that either Kti12 multimerises or that it co-elutes with other unidentified proteins under those conditions. Alternatively, the weak complex between the different proteins might be dissociating during the course of the experiment, resulting in their slight separation.

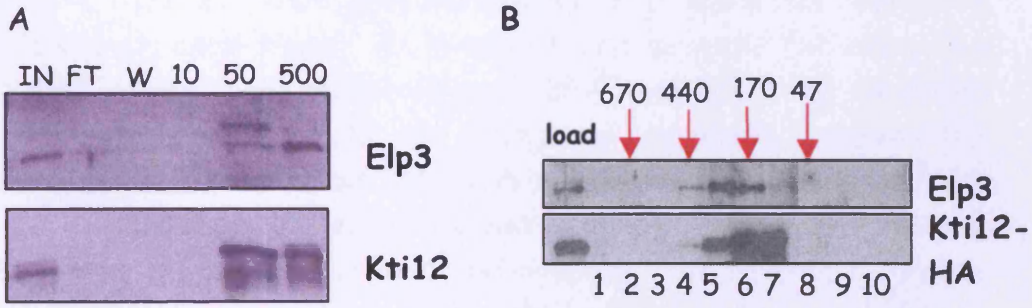

Figure 4.3. Kti12 co-elutes with Elongator under low salt conditions. ***A,*** Western blot analysis of Elp3 and Kti12 following the elution from Ni-agarose. The numbers indicate the mM of imidazole that was used for the elution. IN, input (Ni-agarose load), FT, Ni-agarose flow through, W, wash. Note that Elp3 is represented by the lower band on the blot. The upper band of lane "50" is of unknown origin. ***B,*** Western blot analysis for Elp3 and Kti12 obtained from every second elution fraction of the sizing column. The arrows at the top indicate the elution of protein markers.

Interestingly, under low salt conditions, the elution fractions from 12CA5 beads (2nd step of the purification scheme) also contained

the Rpb1 subunit of RNA polymerase II and the Med1 subunit of the Mediator complex, in a salt-dependent manner (Fig. 4.4A, compare fractions A at 600 mM and 250 mM). Moreover, all the three proteins (Kti12, Rpb1, Med1) seemed to co-elute even after the last step of purification (Ni-agarose) (Fig. 4.4B, lanes "50" and "500"). Although an interaction between Kti12 and RNA polymerase II has been described previously (Fichtner et al., 2002b), the observed interaction with the Mediator subunit Med1 was novel and potentially interesting.

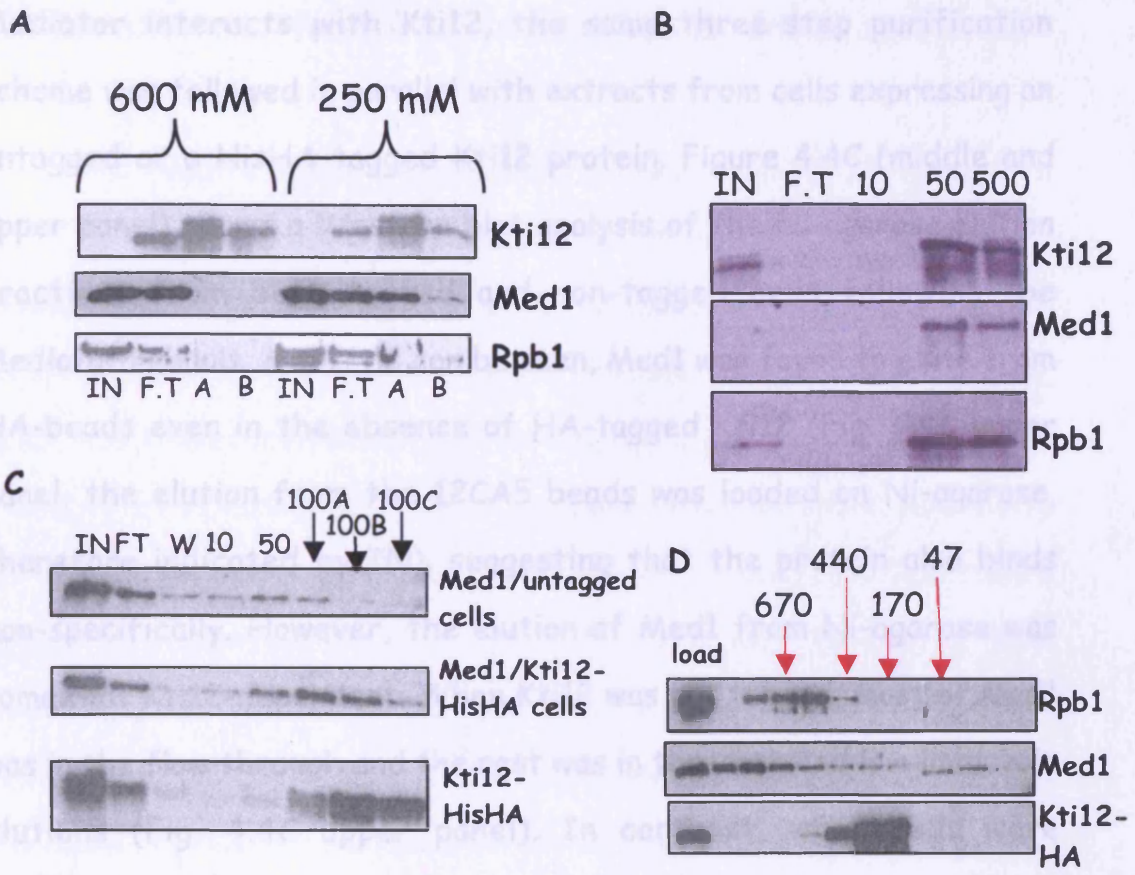

Figure 4.4. Kti12, RNA polymerase II and Mediator co-elute under low salt conditions. *A,* Western blot anlaysis following the second step of purification (binding on 12CA5 beads). Kti12 co-elutes with Rpb1 and Med1 only under low stringency conditions (compare the blots for Rpb1 and Med1 from elution A at 600 mM with the ones from elution A at 250 mM). *B,* Western blot analysis for Kti12, Med1 and Rpb1 obtained from the elution of Ni-agarose. The numbers indicate the concentration of imidazole (in mM) used for the elution. *C,* Extracts from cells expressing HisHA-tagged or untagged Kti12 were subjected to three-step purification. The blot represents a comparison of the elution profile of the indicated proteins from Ni-agarose with increasing amounts of imidazole. *D,* The proteins found in the 500 mM elution from (B) were subjected to sizing column chromatography. Western blot analysis for Rpb1, Med1 and Kti12-HisHA obtained from every second elution fraction of the sizing column. The arrows indicate the elution of the indicated marker proteins.

However, it is often observed that a large multi-subunit complex, such as Mediator, binds non-specifically on different resins under low salt conditions. So, to further test the possibility that

Mediator interacts with Kti12, the same three-step purification scheme was followed in parallel with extracts from cells expressing an untagged or a HisHA tagged Kti12 protein. Figure 4.4C (middle and upper panel) shows a Western blot analysis of the Ni-agarose elution fractions from both tagged and non-tagged cells following the Mediator subunit, Med1. As can be seen, Med1 was found to elute from HA-beads even in the absence of HA-tagged Kti12 (Fig. 4.4C upper panel, the elution from the 12CA5 beads was loaded on Ni-agarose, therefore indicated by IN), suggesting that the protein also binds non-specifically. However, the elution of Med1 from Ni-agarose was somewhat Kti12-dependent. When Kti12 was not tagged, most of Med1 was in the flow through and the rest was in the wash and low-imidazole elutions (Fig. 4.4C upper panel). In contrast, when cells were expressing a Kti12-HisHA protein, some Med1 was found in the flow through but the rest eluted in high imidazole, partly overlapping with the elution of Kti12 (Fig. 4.4C, compare middle with lower panel). This difference, though not very prominent, supports the idea that there is a weak interaction between Kti12 and the Mediator complex.

Finally, the 500 mM imidazole elution fraction from Ni-aagrose shown in Fig. 4.4B, was analyzed by gel filtration chromatography. The elution profile of the sizing column clearly showed that Kti12 did not co-elute with either RNA polymerase II or Mediator (Fig. 4.4D), suggesting that these proteins do not form a stable complex, but merely interact.

4.4 Genetic and functional interaction of Kti12 and Gcn5

The results above indicate that Elongator and Kti12 interact *in vitro*. If Kti12 is also a functional partner of Elongator in the cell, it should interact genetically with Gcn5, as Elongator does. The genetic interaction between Kti12 and the HAT subunit of the SAGA complex, Gcn5, was therefore tested. The *gcn5Δ elp3Δ* double mutant displays a number of severe growth defects, such as temperature sensitivity (37°C) (Wittschieben et al., 2000). Therefore, a *gcn5Δ kti12Δ* double mutant was constructed and its growth at 37°C was tested. Fig. 4.5A shows that the *gcn5Δ kti12Δ* mutant cells are also sensitive to high temperature (37°C). Moreover, the triple mutant lacking *KTI12*, *GCN5* and *ELP3* showed the same sensitivity as the *gcn5Δ elp3Δ*, as judged by the sensitive drop test of Fig. 4.5B, suggesting that Kti12 and Elp3 participate in the same genetic pathway. Interestingly, additional deletion of two genes encoding histone de-acetylases (*HOS2* and *HDA1*) from *gcn5Δ kti12Δ* cells restored the sensitivity to elevated temperature to wild type levels (Fig. 4.5C). Thus, as previously shown for *elp3Δ gcn5Δ hos2Δ hda1Δ* (Wittschieben et al., 2000), *kti12Δ gcn5Δ hos2Δ hda1Δ* are also capable of growing at 37°C, providing further evidence for a functional interaction between Kti12 and Elp3.

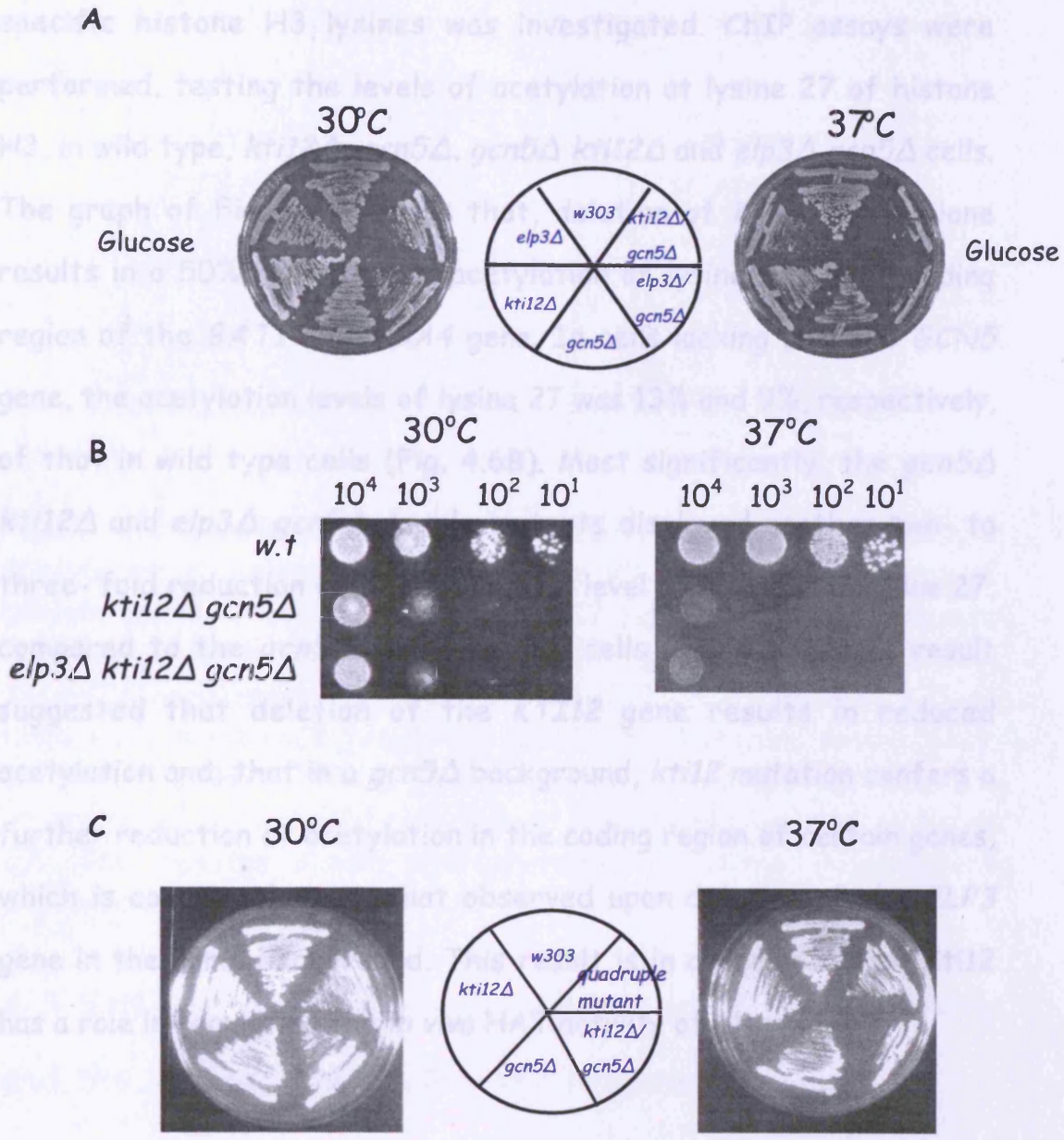

Figure 4.5. Genetic interaction between Kti12 and Gcn5. ***A, C,*** Cells from the indicated strains were streaked on YPD plates and grown for 3-5 days at 30°C or 37°C, as indicated. ***B***, Serial dilutions of cells from the indicated strains were spotted on YPD plates and left to grow for 3-5 days at 30°C or 37°C.

Kristjuhan et al. (2002), previously showed that the severe growth phenotypes of the *elp3Δ gcn5Δ* double mutant cells correlates with hypo-acetylation of histone H3 in the coding region of a number of different genes. Inspired by this study, the possibility that the *gcn5Δ kti12Δ* double mutant cells also display hypo-acetylation of

specific histone H3 lysines was investigated. ChIP assays were performed, testing the levels of acetylation at lysine 27 of histone H3, in wild type, *kti12Δ*, *gcn5Δ*, *gcn5Δ kti12Δ* and *elp3Δ gcn5Δ* cells. The graph of Fig. 4.6A shows that, deletion of *KTI12* gene alone results in a 50% reduction of acetylation of lysine 27 in the coding region of the *BAT1* and *SSA4* gene. In cells lacking only the *GCN5* gene, the acetylation levels of lysine 27 was 13% and 9%, respectively, of that in wild type cells (Fig. 4.6B). Most significantly, the *gcn5Δ kti12Δ* and *elp3Δ gcn5Δ* double mutants displayed another two- to three- fold reduction of the acetylation level of histone H3 lysine 27, compared to the *gcn5Δ* single mutant cells (Fig. 4.6B). This result suggested that deletion of the *KTI12* gene results in reduced acetylation and, that in a *gcn5Δ* background, *kti12* mutation confers a further reduction of acetylation in the coding region of certain genes, which is comparable with that observed upon deletion of the *ELP3* gene in the same background. This result is in agreement that Kti12 has a role in regulating the *in vivo* HAT activity of Elongator.

A B

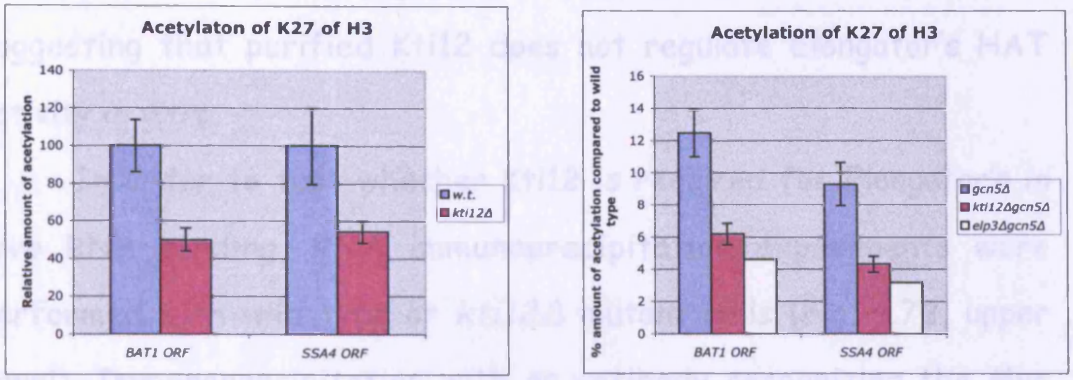

Figure 4.6 *gcn5Δ kti12Δ* double mutant cells display reduced acetylation of lysine (K) 27 of histone H3 in the coding region of certain genes. ***A,*** Deletion of the *KTI12* gene results in a moderate reduction of acetylation compared to wild type cells. ***B,*** Cells lacking the *GCN5* gene retain only 13% and 9% of the acetylation of K27 of histone H3 in the coding region of *BAT1* and *SSA4* gene, respectively. Deletion of either *KTI12* or *ELP3*, in the *gcn5Δ* background, results in an additional 2- to 3-fold reduction of the acetylation level of lysine 27. The results shown are averages of three independent experiments.

4.5 Kti12 is dispensable for the *in vitro* HAT activity and the *in vivo* RNA binding of Elongator

Based on the described physical, genetic and functional interaction between Elongator and Kti12, it was relevant to study whether Kti12 has an effect on the *in vitro* HAT and the *in vivo* RNA binding activity of Elongator. Therefore, *in vitro* HAT assays were performed using highly purified Elongator with or without the addition of increasing amounts of the highly purified Kti12 (depicted in Fig. 4.2). As expected, holo-Elongator acetylated histone H3, whereas Kti12 had no HAT activity on its own (Fig. 4.7A, compare lane 1 with lanes 4, 5). No obvious dramatic effect (stimulatory or inhibitory) was

detected when increasing amounts of Kti12 were added in the presence of Elongator (Fig. 4.7A, compare lane 1 with lanes 2, 3), suggesting that purified Kti12 does not regulate Elongator's HAT activity *in vitro*.

In order to test whether Kti12 is required for Elongator's *in vivo* RNA binding, RNA immunoprecipitation experiments were performed with wild type or *kti12Δ* mutant cells (Fig. 4.7B, upper panel). Immunoprecipitation with an antibody recognizing the Myc epitope from cells expressing Myc-tagged, but not untagged, Elp1 was found to efficiently co-immunoprecipitate mRNA derived from transcription of the *GAL1* gene. In the absence of *KTI12* gene, the levels of RNA bound to Elp1 were slightly reduced, but Elp1 clearly retained its ability to bind *GAL1* mRNA, suggesting that Kti12 has little, if any, role in Elongator's RNA-binding activity. In Fig. 4.7B, the middle panel is a Western blot analysis, which shows that the Elp1 protein levels in *kti12Δ* cells were similar to those in wild type (the lower panel is a Ponceau S stained membrane used as a loading control). Taken together, these results suggest that the Kti12 protein is dispensable for the integrity, the *in vitro* HAT activity and the *in vivo* RNA binding activity of Elongator. However, our data also suggest that Kti12 regulates Elongator's HAT activity *in vivo*, presumably *via* a direct interaction.

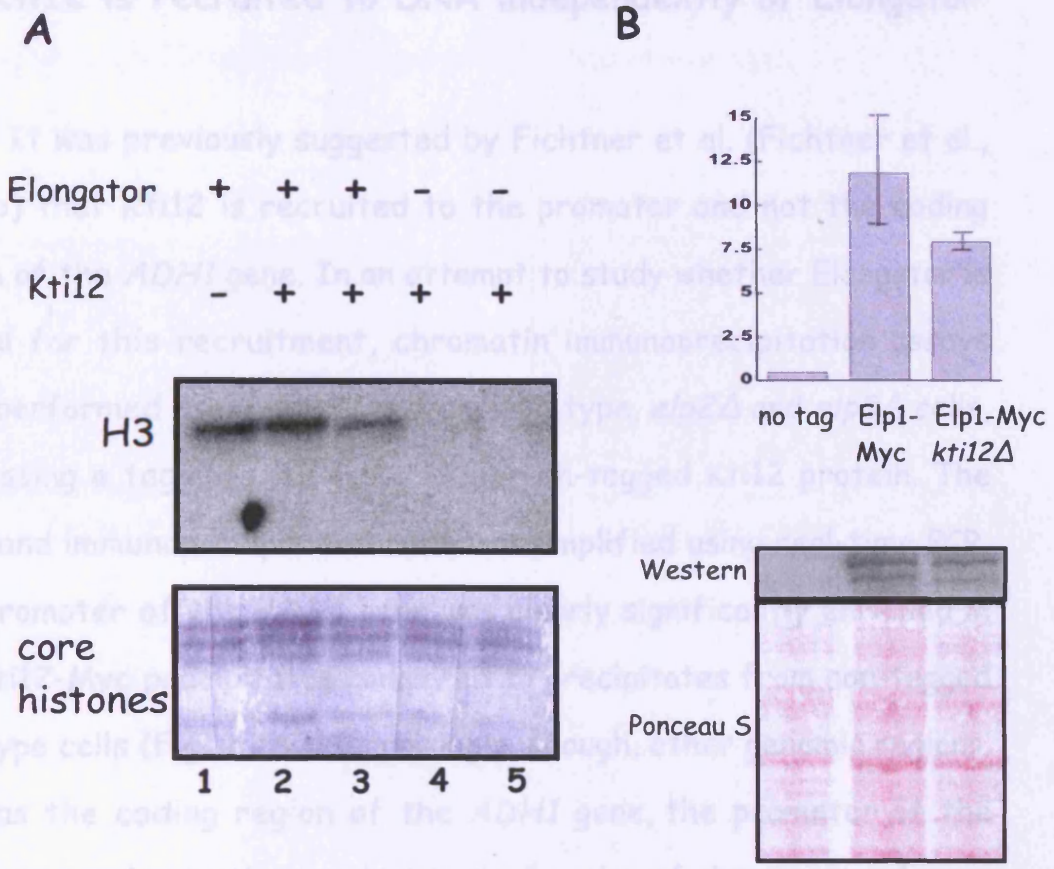

Figure 4.7 Kti12 does not affect the *in vitro* HAT activity or the *in vivo* RNA binding of Elongator. ***A***, *In vitro* HAT assays using highly purified Elongator and/or Kti12. The upper panel is a fluorogram of ^{3}H labelled histone H3 and the lower panel is a coomassie staining of the core histones that were used as a substrate. In lane 1 and 2, approximately 2.8 pmoles of Elongator were used, whereas in lane 3 only 2 pmoles. In lane 2 and 4, 2.8 pmoles of purified Kti12 were used. In lane 3 and 5, the amount of Kti12 was 5.6 pmoles. Note that in lane 3 the reduced acetylation of H3 coincides with the reduced amount of purified Elongator that was used. ***B***, RNA immunoprecipitation (RIP) from wild type cells carrying non-tagged or Myc-tagged Elp1, and from *kti12Δ* cells expressing Myc-tagged Elp1 protein. The middle panel is a western blot analysis of the extracts that were used for the RIP, using an anti-Myc antibody. The lower panel is a Ponceau S stained membrane used as a loading control of the Western blot. The resolution of Elp1 into a double band has been described previously (Fichtner et al., 2003).

4.6 Kti12 is recruited to DNA independently of Elongator

It was previously suggested by Fichtner et al. (Fichtner et al., 2002b) that Kti12 is recruited to the promoter and not the coding region of the *ADH1* gene. In an attempt to study whether Elongator is crucial for this recruitment, chromatin immunoprecipitation assays were performed using extracts from wild type, *elp2Δ* and *elp3Δ* cells, expressing a tagged (Kti12-Myc18), or un-tagged Kti12 protein. The input and immunoprecipitated DNA was amplified using real-time PCR. The promoter of the *ADH1* gene was clearly significantly enriched in the Kti12-Myc precipitates compared to precipitates from non-tagged wild type cells (Fig. 4.8A). Surprisingly, though, other genomic regions, such as the coding region of the *ADH1* gene, the promoter of the *FBA1* gene and even the non-transcribed region of chromosome 4 were also found to be enriched in these precipitates (Fig. 4.8B). It should be mentioned that this applied to all the genomic regions that were tested. This surprising observation suggests that Kti12 is more or less globally associated with chromatin rather than specifically with the promoter of a gene as suggested by the results of Fichtner et al. (Fichtner et al., 2002b).

The use of cells lacking either the *ELP2* or *ELP3* gene was relevant for two reasons: (i) it was suggested by Fichtner et al. (Fichtner et al., 2002b) that Elp2 might interact with Kti12, and (ii) Elp3 is crucial for the integrity of Elongator (Fig. 3.4). Importantly, deletion of either of those genes had no significant effect on the recruitment of Kti12 to the tested genomic regions (Fig. 4.8C). It should be mentioned that the expression level of Kti12 was not affected by the deletion of either *ELP2* or *ELP3* gene (data not

shown). Therefore, these results suggest that Kti12 is recruited to chromatin in an Elongator- and gene-independent manner.

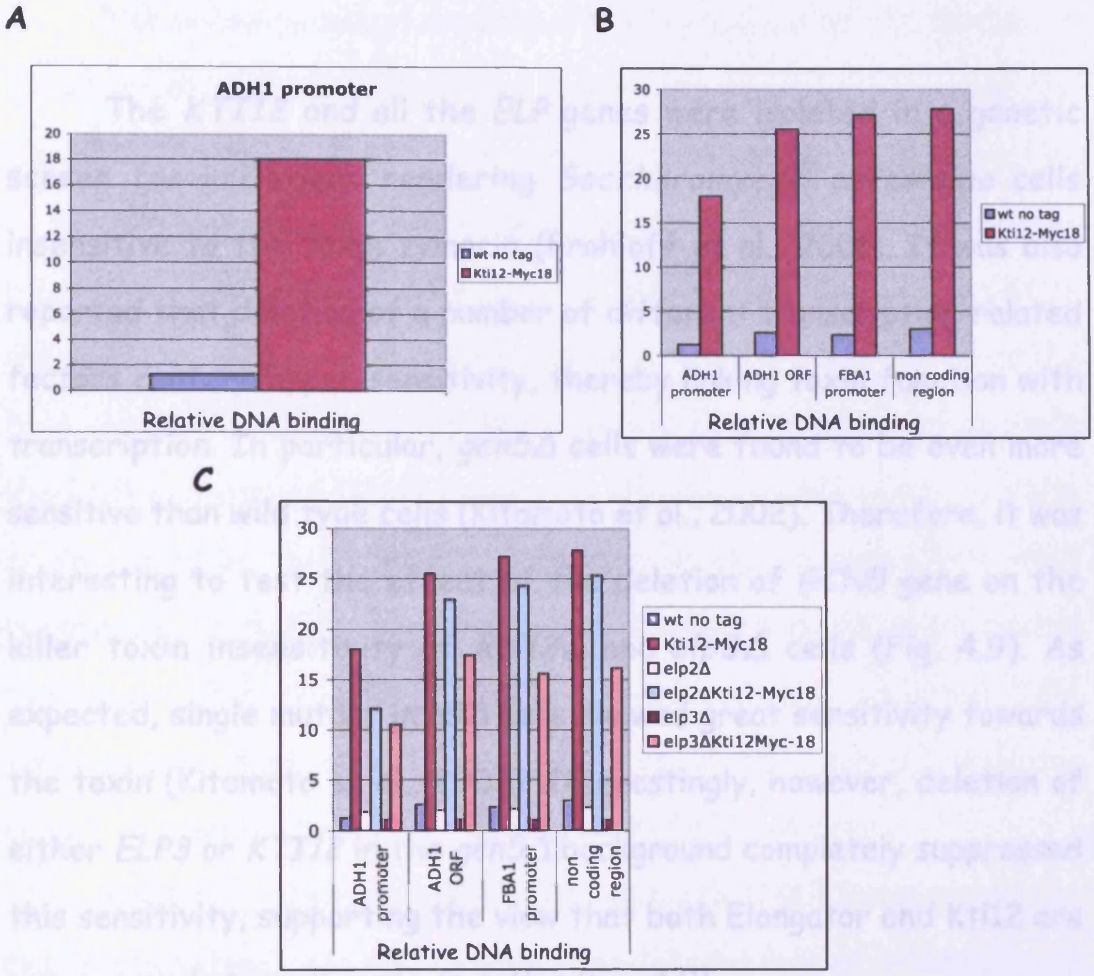

Figure 4.8 Kti12 is recruited to several regions of genomic DNA. ***A,*** Chromatin immunoprecipitation with an anti-Myc antibody (9E11) using extracts from cells expressing un-tagged or Myc-tagged Kti12. The immunoprecipitated DNA was quantitated by real-time PCR with normalization to the amount of total input DNA ("Relative DNA binding"). ***B,*** Immunoprecipitation of Kti12-Myc18 to different regions of the genome. ORF stands for "open reading frame". ***C,*** Deletion of *ELP2* or *ELP3* gene does not affect the recruitment of Kti12 to DNA. Chromatin immunoprecipitation using extracts from wild type, *elp2Δ* and *elp3Δ* cells. Specific primers for the promoter and the coding region of *ADH1* gene (ORF), the promoter of *FBA1* gene, and the non-transcribed region of chromosome 4 were used for the amplification of DNA from Myc-immunoprecipitates.

4.7 Interplay between different factors in zymocin's mechanism of action

The *KTI12* and all the *ELP* genes were isolated in a genetic screen for mutations rendering *Saccharomyces cerevisiae* cells insensitive to the toxin zymocin (Frohloff et al., 2001). It was also reported that deletion of a number of different transcription-related factors confers hyper-sensitivity, thereby linking toxin function with transcription. In particular, *gcn5Δ* cells were found to be even more sensitive than wild type cells (Kitamoto et al., 2002). Therefore, it was interesting to test the effect of the deletion of *GCN5* gene on the killer toxin insensitivity of *kti12Δ* and *elp3Δ* cells (Fig. 4.9). As expected, single mutant *gcn5Δ* cells showed great sensitivity towards the toxin (Kitamoto et al., 2002). Interestingly, however, deletion of either *ELP3* or *KTI12* in the *gcn5Δ* background completely suppressed this sensitivity, supporting the view that both Elongator and Kti12 are primary mediators of zymocin action (Fig. 4.9).

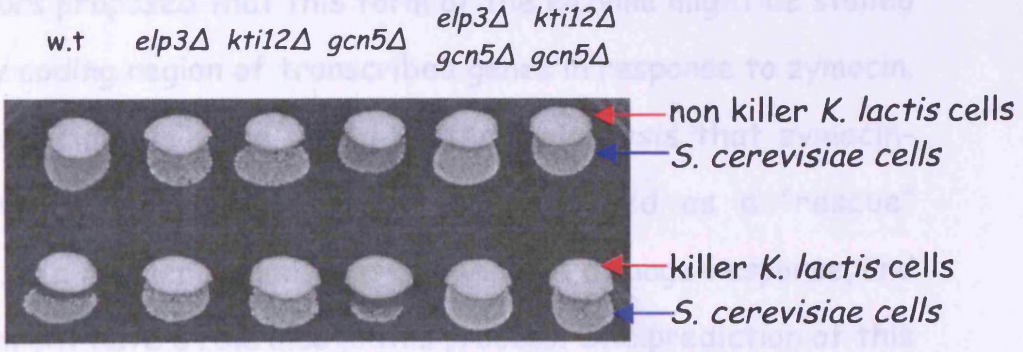

Figure 4.9 Deletion of *ELP3* or *KTI12* suppresses the killer toxin sensitivity of *gcn5Δ* cells. Killer eclipse assays; killer or non killer *K. lactis* cells (red arrows) were grown o/n on a YPD plate at 30 °C. The next day, *S. cerevisiae* cells (blue arrows) from the indicated strains were spotted in the vicinity of the *K. lactis* cells. Toxin-sensitive wild type and hypersensitive *gcn5Δ* cells show an eclipse-like growth. Both

elp3Δ and *kti12Δ* mutant cells are insensitive. Deletion of either *ELP3* or *KTI12* completely suppresses the sensitivity of *gcn5Δ* cells.

The following set of experiments was inspired by the studies of Woudstra et al., (2002). In these studies the yeast homologue of the human CSB protein, Rad26, was purified from the chromatin fraction of yeast cell extracts. Rad26 was found to interact with another polypeptide, called Def1 (Woudstra et al., 2002). The phenotypes of cells lacking *DEF1* are consistent with a role for this factor in the DNA damage-response. In contrast to wild-type cells, which degrade the RNA polymerase II in response to UV, *def1Δ* cells were unable to do so. Based on their results, Woudstra et al. suggested that RNA polymerase II stalled at a DNA lesion triggers a coordinated rescue mechanism that requires the Rad26-Def1 complex, and that Def1 enables ubiquitination and proteolysis of RNAPII when the lesion cannot be rapidly removed by Rad26-promoted DNA repair.

Strikingly, Jablonowski and Schaffrath (2002) observed that the protein level of the un-phosphorylated form of RNAPII was reduced in response to zymocin. Moreover, based on other results these authors proposed that this form of the enzyme might be stalled in the early coding region of transcribed genes in response to zymocin. My next experiments were based on the hypothesis that zymocin-stalled RNA polymerase II might be degraded as a "rescue" mechanism, in a manner reminiscent of the DNA damage-response, and that Def1 might have a role also in this process. One prediction of this model was that *def1Δ* cells would be expected to be very sensitive to the toxin. Indeed, the eclipse assays of Fig. 4.10A shows that *def1Δ* cells are extremely zymocin sensitive, much more than wild type cells. Deletion of the gene, which encodes the biochemical partner of Def1,

Rad26, showed wild type sensitivity. Interestingly, the double mutant *def1Δ rad26Δ* cells were as sensitive as the single *def1Δ* mutant cells, suggesting a Rad26-independent role for Def1 in zymocin's mechanism of action. Additionally, when the *ELP3* gene was deleted in a *def1Δ* background, the cells were zymocin insensitive suggesting that the role of Elp3 in zymocin action precedes that of Def1.

The above genetic data, which suggest a possible involvement of Def1 in the cellular response to zymocin, raised the question of whether other factors participating in cellular response to UV might also play a role in zymocin toxicity. The zymocin sensitivity of a number of different deletion mutants for genes involved in DNA repair was tested. Deletion of either *RAD16* or *RAD18*, which encode proteins with roles in global genome repair and damage tolerance/post-replication repair pathways, respectively, conferred wild type sensitivity in killer eclipse assays (data not shown). Interestingly, however, *rad14Δ* cells were killer-toxin insensitive (Fig. 4.10B). Rad14 and its human homologue, XPA, recognise damaged DNA and are key components in the nucleotide excision repair pathway (Prakash and Prakash, 2000). However, cells with deletion of genes that encode other members of the same pathway, like *RAD1* and *RAD10*, behaved like wild type cells, indicating a possible NER-independent role for Rad14 (Fig. 4.10B). Intriguingly, the *def1Δ rad14Δ* double mutant cells displayed wild type toxin sensitivity (Fig. 4.10B), suggesting that in the absence of both Rad14 and Def1 the toxin still functions, possibly by using an alternative pathway.

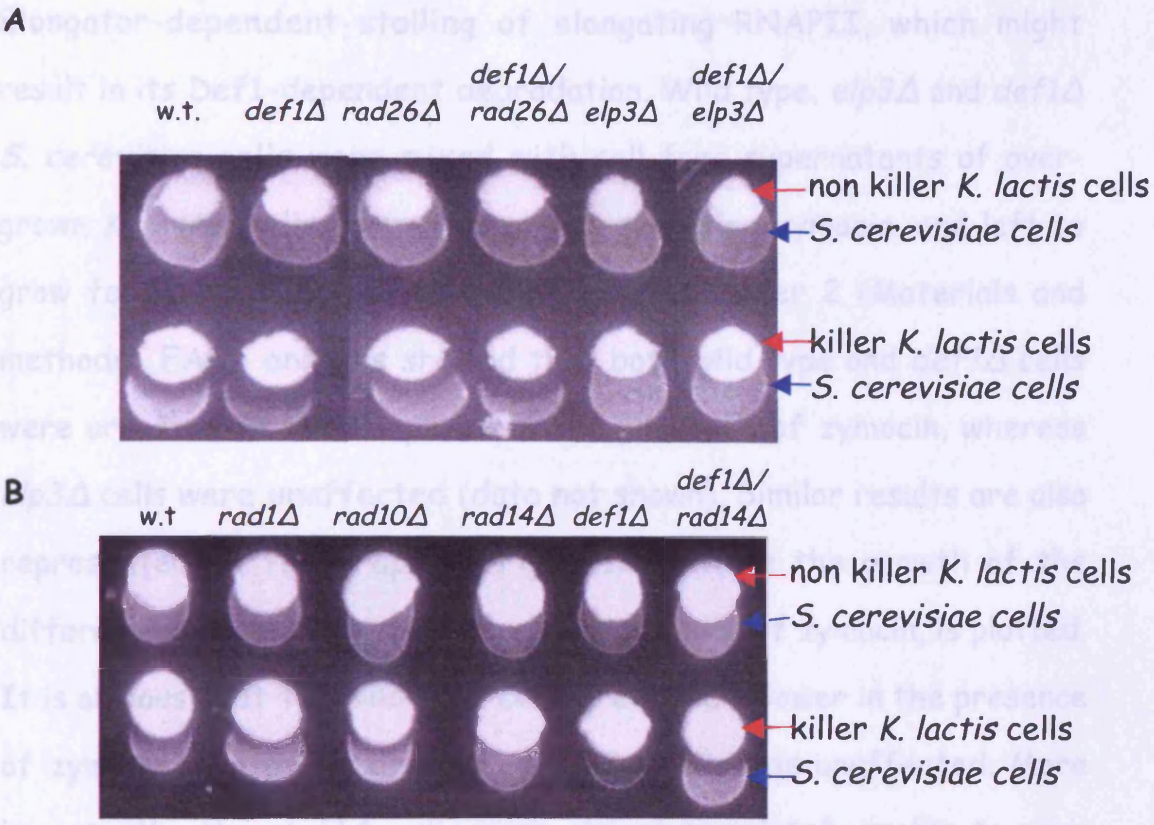

Figure 4.10 Killer eclipse assays testing the sensitivity of DNA repair-related mutant cells in zymocin's mechanism of action. *A,* Killer eclipse assays showing that *def1Δ* mutant cells are extremely sensitive towards zymocin. *rad26Δ* cells display wild type sensitivity, whereas the sensitivity of *def1Δ rad26Δ* double mutant cells resembles that of the *def1Δ* cells. Moreover, deletion of *ELP3* suppressed the sensitivity of *def1Δ* cells. *B,* Mutants of NER-related factors tested for zymocin sensitivity (*rad1Δ, rad10Δ* and *rad14Δ*). *rad14Δ* cells are insensitive, whereas *rad1Δ* and *rad10Δ* cells display wild type sensitivity. Deletion of *DEF1* in *rad14Δ* cells re-stored wild type sensitivity.

4.8 The effect of zymocin on RNA polymerase II

Inspired by the genetic data, which showed that *elp3Δ* cells are insensitive to zymocin while *def1Δ* cells are extremely sensitive (Fig. 4.10A), we decided to investigate whether the level of sensitivity correlated with the protein levels of the un-phosphorylated form of RNA polymerase II. We hypothesized that zymocin might lead to

Elongator-dependent stalling of elongating RNAPII, which might result in its Def1-dependent degradation. Wild type, *elp3Δ* and *def1Δ* *S. cerevisiae* cells were mixed with cell-free supernatants of over-grown *K. lactis* cells, secreting or not secreting zymocin, and left to grow for up to 6 hours, as described in Chapter 2 (Materials and methods). FACS analysis showed that both wild type and *def1Δ* cells were arrested in the G1 phase in the presence of zymocin, whereas *elp3Δ* cells were unaffected (data not shown). Similar results are also represented on the graph of Fig. 4.11A, where the growth of the different cell types, in the presence or absence of zymocin, is plotted. It is obvious that the wild type cells grew much slower in the presence of zymocin, while the growth of *elp3Δ* cells was unaffected. More importantly, the *def1Δ* cells were almost completely unable to grow after the addition of zymocin-containing medium. These rates of growth thus correlated exactly with the results of the eclipse assays (Fig. 4.10A).

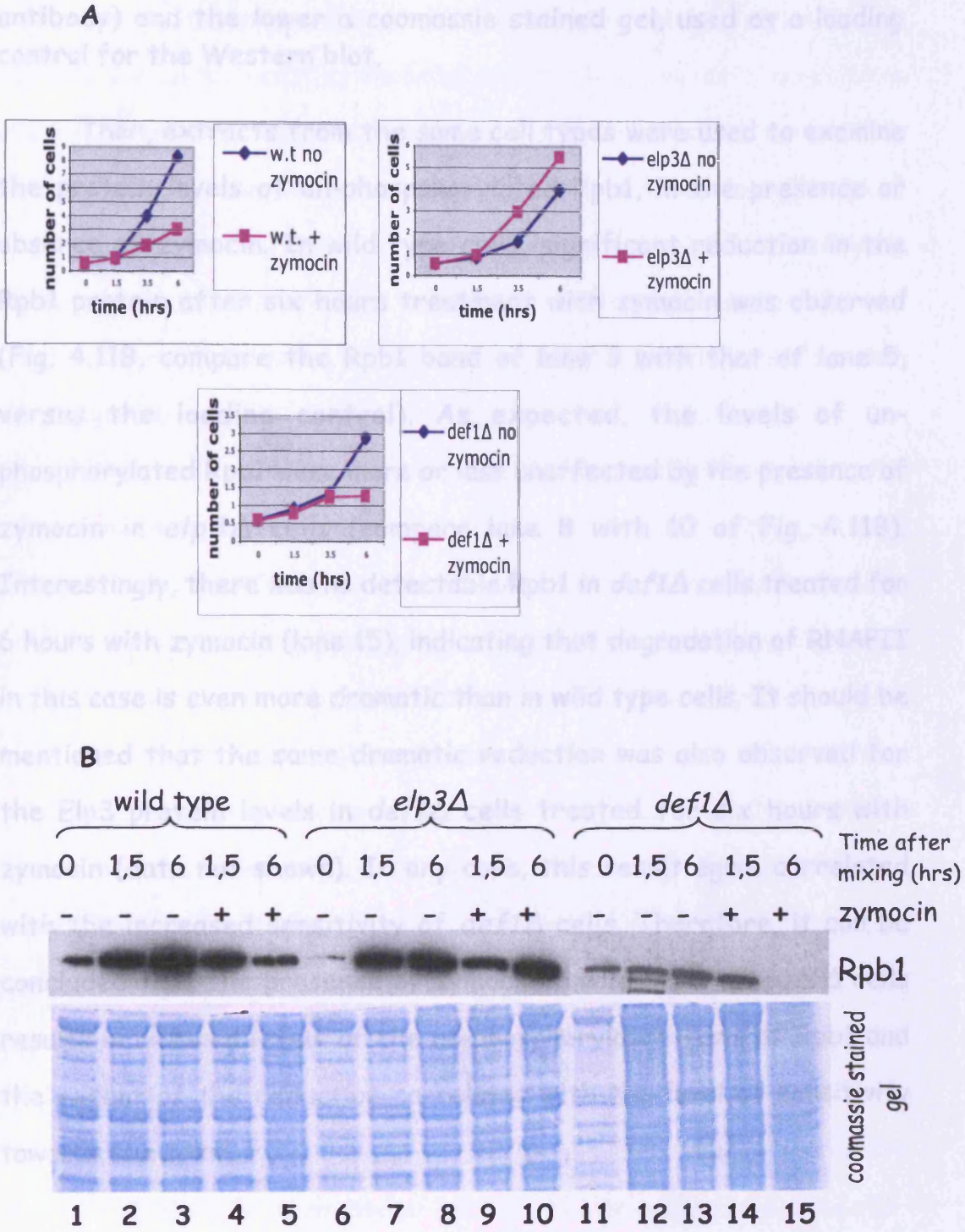

Figure 4.11 Zymocin effect on growth rate and protein levels of un-phosphorylated Rpb1 in different cell types. **A,** Wild type, *elp3Δ* and *def1Δ* cells were grown in the presence or absence of zymocin, and their growth rate was plotted on a diagram. The number of cells is indicated in 10^6. **B,** Western blot analysis using extracts from the cells that were used for the growth rate assay. The upper panel is a Western blot analysis for un-phosphorylated Rpb1 (8WG16

antibody) and the lower a coomassie stained gel, used as a loading control for the Western blot.

Then, extracts from the same cell types were used to examine the protein levels of un-phospshorylated Rpb1, in the presence or absence of zymocin. In wild type cells, significant reduction in the Rpb1 protein after six hours treatment with zymocin was observed (Fig. 4.11B, compare the Rpb1 band of lane 3 with that of lane 5, versus the loading control). As expected, the levels of un-phosphorylated Rpb1 were more or less unaffected by the presence of zymocin in *elp3Δ* cells (compare lane 8 with 10 of Fig. 4.11B). Interestingly, there was no detectable Rpb1 in *def1Δ* cells treated for 6 hours with zymocin (lane 15), indicating that degradation of RNAPII in this case is even more dramatic than in wild type cells. It should be mentioned that the same dramatic reduction was also observed for the Elp3 protein levels in *def1Δ* cells treated for six hours with zymocin (data not shown). In any case, this result again correlated with the increased sensitivity of *def1Δ* cells. Therefore, it can be concluded that the presence of zymocin in wild type, or *def1Δ* cells results in reduced levels of the un-phosphorylated form of Rpb1 and the extent of the reduction correlates with the level of sensitivity towards the toxin.

4.9 Discussion and conclusions

KTI12 and all the *ELP* genes were isolated in a genetic screen for mutations rendering *Saccharomyces cerevisiae* cells insensitive to zymocin (Frohloff et al., 2001). Subsequently, Schaffrath and co-workers showed that Elongator interacts with Kti12 in co-

immunoprecipitation experiments. However, the integrity of the six-subunit Elongator complex remains intact when the Kti12 protein is missing (Fichtner et al., 2002a). Kti12 was also shown to interact with RNAPII, in particular with the serine 5 phosphorylated form (Frohloff et al., 2003). Furthermore, *kti12Δ* mutant cells displayed phenotypes that are very similar to *elpΔ* mutants, including sensitivity to 6-AU. These observations indicated that Kti12 might be a functional partner of Elongator.

The results presented in the first part of this Chapter represent the first comprehensive analysis of the interaction between Elongator and Kti12 and the possible role for this interaction *in vivo* and *in vitro*. Using co-immunoprecipitation assays the previously reported interaction between Elongator and Kti12 was confirmed. However, this interaction was stable only under low salt conditions (Fig. 4.1). This observation suggests that Elongator interacts with, but is not stably associated with, Kti12. Moreover, yeast Kti12 was purified as a single protein from the soluble fraction of extracts under stringent conditions, whereas under low stringency a sub-population of Elongator seemed to co-purify with a substantial amount of Kti12 (Fig. 4.2, Fig. 4.3). However, the stoichiometry of this complex is unknown. The highly purified yeast Kti12 protein presented in Fig. 4.2 did not have any effect on the *in vitro* histone acetyl-transferase activity of Elongator under the conditions tested (Fig. 4.4). In addition, deletion of *KTI12* gene did not affect the *in vivo* RNA binding of Elongator (Fig. 4.4). These results indicated that the Kti12 does not directly regulate the *in vitro* HAT activity or the *in vivo* RNA binding of Elongator.

Genetic studies, on the other hand, suggested a functional interaction between Kti12 and Gcn5 reminiscent of that between Elp3 and Gcn5 (Fig. 4.5), supporting the idea that Kti12 affects *in vivo* Elongator function. Thus, the *kti12Δ gcn5Δ* double mutant displayed increased sensitivity to high temperature (37°C), a characteristic previously reported for *elp3Δ gcn5Δ* cells (Wittschieben et al., 2000). Moreover, the acetylation levels of lysine 27 of histone H3 in the coding region of the *BAT1* and *SSA4* gene (two of the genes that showed reduced acetylation in *elp3Δ gcn5Δ* cells (Kristjuhan et al., 2002)) were examined in *kti12Δ gcn5Δ* cells. Fig. 4.6 shows that the concomitant deletion of *KTI12* and *GCN5* resulted in a reduction of the acetylation levels comparable to that observed upon deletion of both *ELP3* and *GCN5*. Since Kti12 interacts with Elongator, this result is most easily understood if there is a defect in the *in vivo* HAT activity of Elongator in the absence of the *KTI12* gene. It should be mentioned that this is the first time that a possible *in vivo* functional relationship between Elongator and Kti12 has been demonstrated. To obtain more support for a regulatory role of Kti12 in the *in vivo* HAT activity of Elongator, it would be of great interest if point mutations could be identified that disrupt the interaction between Elongator and Kti12. If Kti12 regulates the *in vivo* HAT activity of Elongator by direct protein-protein interactions, these mutations should have the same effect as deletion of the entire *KTI12* gene on the acetylation levels of the previously mentioned genes.

In sharp contrast to data obtained by Fichtner et al. (Fichtner et al., 2002b), who reported recruitment of Kti12 protein only to the promoter of the *ADH1* gene, the ChIP experiments presented in this Chapter suggest that the Kti12 is present in several regions along the

genome, such as coding regions and even un-transcribed sequences. Moreover, the presence of Kti12 in these regions was independent of Elongator. This was a surprising result. One obvious explanation is that Kti12 is a component of chromatin. It has recently been shown that activation of the *GAL1* gene results in reduced histone DNA contacts (nucleosome loss) at the gene (Kristjuhan and Svejstrup, 2004). If Kti12 associates with chromatin (rather than being a DNA-binding protein), galactose induction would also be expected to result in reduced detection of Kti12 protein in the coding region of *GAL* genes. This idea is currently under investigation.

In the second part of this Chapter, the mode of action of the killer toxin zymocin was investigated. In particular, the idea that zymocin action involves degradation of RNAPII was tested. Zymocin is secreted from *K. lactis* cells and results in the arrest of wild type *S. cerevisiae* cells in the G1 phase of the cell cycle. Work from Schaffrath and co-workers suggested that RNA polymerase II-dependent transcription is impaired in the presence of zymocin. Moreover, it was shown that the protein levels of the un-phosphorylated Rpb1 subunit are dramatically reduced (Jablonowski and Schaffrath, 2002). In the same report, chromatin immunoprecipitations were used to study the recruitment of RNAPII to the *ADH1* gene in the presence or absence of zymocin. It was suggested that zymocin results in reduction of the un-phosphorylated form of RNA polymerase II at the promoter, and that it is instead stably bound at the beginning of the gene. The proposed interpretation of these results was that in the presence of zymocin the un-phosphorylated form of RNAPII is stalled in the beginning of the gene (Jablonowski and Schaffrath, 2002).

Elongator seems to be one of the primary targets of zymocin and a pre-requisite of its function in *S. cerevisiae* cells, since any tested combination of deletion mutants that include an *ELP3* deletion show the same lack of sensitivity that is observed in single *elp3Δ* mutant cells. Most likely, the same is true for Kti12 (Fig. 4.9).

Inspired by the model proposed by Jablonowski and Schaffrath (2002), I speculated that zymocin-induced stalled RNAPII might be subjected to degradation in a way similar to that by which RNAPII, stalled on damaged DNA, is thought to be removed (Woudstra et al., 2002). Based on this idea, the toxin sensitivity of *def1Δ* cells was tested. Def1 is the only protein known to specifically be involved in the degradation of RNAPII in the presence of UV-induced damage (Woudstra et al., 2002). According to the above speculation, *def1Δ* cells would be expected to be very sensitive to zymocin, because they would accumulate stalled RNAPII without the ability to degrade it. In the same way, *rad26Δ* cells were expected to be less sensitive than wild type cells, and the *def1Δ rad26Δ* double mutant to regain wild type toxin sensitivity. Figure 4. 10 shows that *def1Δ* cells were indeed very toxin sensitive. However, the *rad26Δ* cells displayed wild type sensitivity and the *def1Δ rad26Δ* double mutant cells behaved like the *def1Δ* cells. Although the possibility that zymocin's way of action involves an alternative mechanism independent of degradation of RNAPII cannot be excluded, the above results might suggest a novel, Rad26-independent, role for Def1 in zymocin's mechanism of action.

A surprising result was obtained from the Western blot analysis examining the change in protein levels of the un-phosphorylated form of Rpb1 in response to zymocin in different cell types. Interestingly, Rpb1 protein levels decreased 6 hours after treatment with zymocin in

wild type cells. In agreement with the insensitivity in the killer eclipse assays, the Rpb1 protein levels were unaffected in *elp3Δ* cells. Intriguingly, and in apparent disagreement with the above speculation about the possible role of Def1 in zymocin action, the amount of un-phosporylated Rpb1 was not stabilized, but rather dramatically reduced, in *def1Δ* cells (Fig. 4.11). In fact, after six hours there was no detectable Rpb1 in *def1Δ* cells. This observation suggests that zymocin results in reduced levels of un-phosphorylated RNAPII, which in turn results in impaired RNAPII function (other proteins also showed reduced levels after 6 hours treatment). Additionally, these results have uncovered a new Def1-independent RNAPII degradation pathway, which is detrimental for growth in the presence of zymocin.

However, the possibility of a secondary effect of zymocin on Rpb1 protein expression cannot be excluded (in the absence of transcription, protein production will eventually cease). One way to distinguish between increased protein degradation and decreased protein production, would be by showing that in wild type and *def1Δ* cells the rate of degradation of RNAPII is increased in the presence of zymocin and cyclohexamide, compared to that in the presence of cyclohexamide alone (which inhibits translation). It might also be informative to investigate the effect of zymocin on Rpb1 protein levels in a proteasome-deficient strain. If the proteasome is involved in degradation of RNAPII in response to zymocin, these cells might be toxin-sensitive, and retain normal Rpb1 protein levels in the presence of the toxin. Finally, it should be mentioned that 1.5 hrs after the treatment with zymocin, the Rpb1 protein levels were comparable to those of untreated cells in both wild type and *def1Δ* cells (compare lanes 2 and 4 and lanes 12 and 14, respectively, of Fig. 4.11B).

Therefore, if zymocin action involves stalling of RNAPII, ChIP analysis of RNAPII in the absence and presence of zymocin at this or even later time points might prove useful. Much work remains to be done before we can hope to understand the complex mechanisms underlying the effect of zymocin on Elongator, transcription, and cell growth.

CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS

The experiments described in the previous two Chapters were designed to address key questions about the composition, the interactions, and the *in vitro* and *in vivo* role of Elongator complex in yeast *S. cerevisiae*. Elongator was initially purified from the insoluble chromatin fraction of yeast extracts as a component of a novel complex, which includes the hyper-phosphorylated form of RNA polymerase II known to be engaged in transcript elongation (Otero et al., 1999). Based on this interaction and genetic evidence, it was suggested that Elongator participates in RNA polymerase II transcript elongation (Fellows et al., 2000; Otero et al., 1999; Wittschieben et al., 1999). Initially, it was thought that Elongator consists of three subunits. However, further biochemical studies using the soluble fraction of yeast extracts uncovered a more complex form of Elongator, consisting of six subunits (Krogan and Greenblatt, 2001; Li et al., 2001; Winkler et al., 2001). The same studies suggested that Elongator can be disrupted into two sub-complexes; core Elongator, composed of the three subunits found initially in the chromatin fraction of extracts, and the "small" sub-complex composed of the three new proteins identified using the soluble fraction of extracts (Krogan and Greenblatt, 2001; Li et al., 2001; Winkler et al., 2001).

The results presented in Figures 3.1 and 3.2 of Chapter 3 further support the notion that the true form of Elongator is a six-subunit complex. In particular, all the six subunits were found to stably associate following the purification of one tagged subunit from each sub-complex (Elp1 or Elp4; Fig.3.1). Additionally, deletion mutants for any of the genes encoding the six Elongator subunits were shown to confer the same growth defects, such as sensitivity to high temperature and high salt (Fig. 3.2 and (Fellows et al., 2000; Otero et

al., 1999; Wittschieben et al., 1999)). Moreover, deletion of two *ELP* genes, one encoding a subunit of core Elongator (*ELP1*) and another encoding a subunit of the "small" sub-complex (*ELP4*), did not show any additional defect, suggesting that these proteins are implicated in the same genetic pathway in the cell (Fig. 3.2).

In an attempt to gain insight into Elongator's molecular architecture, three different approaches were utilized; co-immunoprecipitation assays, yeast two-hybrid interactions and *in vitro* pull down experiments. To better understand the results of co-immunoprecipitation experiments from different *elpΔ* deletion mutants, the stability of Elongator proteins in these mutants was studied. Interestingly, the Elp3 protein was found to be de-stabilized when the Elp1 subunit was missing (Fig. 3.3). That is not surprising, since the protein-protein interaction studies showed a strong, direct interaction between these two subunits, suggesting that Elp3 is more susceptible to degradation when its "partner" is absent (Fig. 3.4). The same protein-protein interaction studies also showed a direct interaction between Elp4 and Elp6 (Fig. 3.5, 3.6) and between Elp5 and Elp6 (Fig. 3.7). Surprisingly, only a weak interaction between Elp4 and Elp3 was observed (Fig. 3.5). Although no direct interaction was observed, there were indications that Elp2 also has a role in stabilising the interaction between the two sub-complexes (Fig. 3.4). However, the fact that Elp2 was not found to play a major role in the formation of the holo-Elongator complex was surprising, since it includes eight WD40 repeats, motifs previously shown to participate in protein-protein interactions. All the identified interactions among the Elongator subunits are summarised in the Fig. 3.8

So far, there is no direct *in vitro* evidence connecting the function of Elongator complex with transcript elongation in yeast. In contrast, human Elongator complex was shown to facilitate transcript elongation through chromatin (Hawkes et al., 2002; Kim et al., 2002). Interestingly, this reaction was dependent on the presence of acetyl-CoA, thus correlating the identified histone acetyl-transferase activity of Elongator with transcript elongation. Moreover, a number of other studies have uncovered an important role of Elongator's HAT activity *in vivo* (Kristjuhan et al., 2002; Wittschieben et al., 2000). For example, it was shown that combined elimination of Elp3 and Gcn5 HAT activity conferred severe growth defects to cells (Wittschieben et al., 2000). This was also the case when deletion of the *GCN5* gene was combined with the deletion of either *ELP1* or *ELP2* (Wittschieben et al., 2000). These defects were shown to correlate with reduced occupancy of RNA polymerase II and overall reduced acetylation levels of histone H3 in the coding region of several genes (Kristjuhan et al., 2002). The interpretation of these results is that Elongator contributes to transcription by acetylating histone H3 of several actively transcribed genes, but that its function is redundant with that of Gcn5.

Elongator can also acetylate core histones and nucleosomes *in vitro* (Winkler et al., 2002). The catalytic subunit is the Elp3 protein and the main target of the activity is histone H3. However, little was known about the role of the other subunits in that function. Winkler et al. showed that biochemically isolated yeast core Elongator is not active as a HAT (Winkler et al., 2002). The same was shown for the human core Elongator complex (hElp1, hElp2, hElp3) (Hawkes et al., 2002). In contrast, the six-subunit holo-Elongator complex was active.

This suggested that the "small" sub-complex is required for the HAT activity of Elongator. To learn more about the role of the individual subunits, "mutant" forms of Elongator complex lacking at least one of the subunits, had to be purified and tested in HAT assays *in vitro*. Unfortunately, that was not generally possible because my experiments showed that Elongator is a fragile complex, which typically dissociates when one of its subunit proteins is absent (Fig. 3.4, 3.5). The only exception was when the Elp2 protein was missing (Fig. 3.4). The co-immunoprecipitation assays of Fig. 3.4 clearly suggested that in the *elp2Δ* cells, the remaining five Elp subunits still form a complex. This complex was therefore purified and compared to Elongator from wild type cells in HAT assays (Fig. 3.9). These experiments suggested that the Elp2 protein is dispensable for the HAT activity of Elongator. It is possible that the WD40 repeats of Elp2 are necessary for interaction with other proteins, such as Kti12, as was suggested by Fichtner et al. (2002b).

Recently, Elongator was shown to have another activity; it binds to RNA both *in vitro* and *in vivo*. Thus, using a modified chromatin immunoprecipitation assay, RNA immunoprecipitation (RIP), Gilbert et al. showed that Elp3, like RNAPII, could be cross-linked to nascent, un-spliced pre-mRNA *in vivo*, and that it is found along the entire length of the transcript (Gilbert et al., 2004). As expected, Elp1 was also found to cross-link to RNA (Fig. 3.10). In an attempt to uncover possible roles for the individual subunits in that RNA association *in vivo*, different *elpΔ* deletion mutants were used, and the efficiency of Elp3 or Elp1 in associating with RNA was compared with that in wild-type cells. Deletion of either *ELP2* or *ELP4* did not have any major effect on Elp3-RNA interaction. Considering that the Elp5 and the

Elp6 do not interact with core Elongator in *elp4Δ* cells (Fig. 3.4), the above result also suggested that the "small" sub-complex is dispensable for the binding of Elp3 to RNA *in vivo*. However, deletion of *ELP3* was shown to dramatically reduce Elongator-RNA interaction *in vivo* (Fig. 3.10). Therefore, Elp3 may have three different roles: critical component for the integrity of holo-Elongator, catalytic subunit for the histone acetyl-transferase activity, and major contributor to RNA binding *in vivo*.

As mentioned previously, Elp3 has two distinct motifs: the HAT motif and the SAM domain. It would, therefore, be of great interest to explore the role of each domain for all the previously mentioned functions. Point mutations, which eliminate the histone acetyl-transferase and the potential histone de-methylase activity, could be introduced and their effect on any of the three mentioned roles of Elp3 could be tested. Finally, it would be extremely interesting to test the possibility that Elp3 has a histone de-methylase activity, as proposed by Chinenov (2000). That can be achieved either by *in vitro* histone methyl-transferase assays/de-methylase assays, or by detecting a possible effect of deletion of *ELP3* gene on the levels of the already known targets for lysine or arginine methylation. Genetic interactions with the SET-domain containing yeast genes would also be indicative for this function. Unpublished work in the Svejstrup laboratory has so far failed to uncover convincing evidence of a role for Elp3 in histone de-methylation.

Although convincing evidence for a role for Elongator in the nucleus has been obtained, most of the localization studies so far clearly suggested that it is found mainly in the cytoplasm, especially in yeast (Fichtner et al., 2002b; Hawkes et al., 2002; Kim et al., 2002;

Pokholok et al., 2002). Recently, however, Elp1 was proposed to have a nuclear localization signal (Fichtner et al., 2003). In an attempt to address the question of cellular localization, the idea that Elongator continuously shuttles from the cytoplasm to the nucleus and back was tested (Fig. 3.11-3.14). In particular, the effect of the inactivation of two factors (Crm1 and Kap120) involved in protein nuclear import/export on the localization of Elp3 was studied. If one of these factors had a role in transferring Elongator (Elp3) from the cytoplasm to the nucleus, elimination of its import function should result in accumulation of Elongator (Elp3) in the nucleus. Unfortunately, no significant accumulation of Elp3 in the nucleus was detected in either case. However, further studies to delineate the localization of Elongator under different conditions would be of great interest. Recently, using GFP staining, Huh et al. (2003) identified approximately 4800 copies of Elp3 in *S. cerevisiae* cells, predominantly found in the cytoplasm (Huh et al., 2003). Identification of a cytoplasmic function for Elongator would seem to be of great importance as well. An intriguing possibility is that it also has a role in acetylating the newly synthesized histones, thereby participating in maintaining the chromosome-wide acetylation levels of the yeast genome.

Finally, the significance of the genetic interaction between Elongator and a number of different elongation factors should be addressed (Kong et al., submitted). This could potentially open up the way of identifying other so far unknown transcription-related functions of the yeast Elongator complex.

The second part of the thesis focuses on the physical and functional connection between Elongator and Kti12. Kti12 was

previously shown, by co-immunoprecipitation experiments, to interact with Elongator, but having no role in maintaining the integrity of the six-subunit holo-Elongator complex. In particular, Fichtner et al. (Fichtner et al., 2002b) suggested that Elp2 might be the subunit that Kti12 interacts with in the Elongator complex. Others, however, have reported that purification of Kti12 from yeast cell extracts resulted in the isolation of a different and seemingly unrelated protein complex (Krogan and Greenblatt, 2001).

The results presented in Chapter 4 showed that Elongator interacts with Kti12, but that this interaction is not stable at high salt concentrations (Fig. 4.1). Moreover, purification of Kti12 from the soluble fraction of yeast extracts, under moderate salt conditions, resulted in its isolation with another polypeptide (Fig. 4.2). However, the other protein was found to be a common contaminant when 12CA5/Ni-agarose tandem purification is used as purification procedure (Gilbert et al., 2003). Under low salt conditions, Kti12 was found to co-elute after three purification steps along with Elongator, RNA polymerase II and Mediator complex (Fig. 4.3, 4.4). However, further characterization suggested that Kti12 is not in a complex with RNA polymerase II and Mediator (Fig. 4.4). Nevertheless, Kti12 was found in Elongator-containing fractions (Fig. 4.3). It seems that Kti12 associates with Elongator in a salt-sensitive complex. It should be mentioned that core Elongator (Elp1, Elp2 and Elp3) - and not holo-Elongator - was recently suggested to co-purify with another protein called Kti11 (Fichtner et al., 2003). The importance of this purification and the possibility that Elongator can form a salt-labile complex with both Kti11 and Kti12 requires further investigation. It is worth mentioning that Kti12 has also been suggested to localize mainly in the

cytoplasm, whereas Kti11 was found to occupy both the cytoplasm and the nucleus (Fichtner et al., 2002b; Fichtner et al., 2003). The fact that Kti12 clearly is localized in the nucleus as judged by chromatin immunoprecipitation assays (Fichtner et al., 2002a), shows that cellular localization data obtained by immuno- or GFP-localization should be regarded with caution. The precise relationship between these proteins and their function in cells is an important issue that merits further investigation.

The results of Fig. 4.5, 4.6 and 4.7 present the first comprehensive *in vitro* and *in vivo* analysis of functional interaction between the Elongator and Kti12. In particular, the purified yeast Kti12 was used in *in vitro* HAT assays to test whether it stimulates (or inhibits) Elongator's ability to acetylate histone H3 (Fig. 4.7). Additionally, the ability of Elp1 to cross-link to RNA in cells lacking the *KTI12* gene was studied (Fig. 4.7). In both cases, Kti12 found not to affect Elongator function. In contrast to that, Kti12 was shown to interact genetically with Gcn5, in a way similar to that of Elp3 (Fig. 4.5). Moreover, deletion of both *KTI12* and *GCN5* resulted in reduction of the acetylation levels of specific lysines in the coding regions of some genes, resembling the phenotype of the double mutant *elp3Δ gcn5Δ* cells (Fig. 4.6). Thus, these experiments suggest a regulatory role for Kti12 in Elongator's *in vivo* HAT activity. In the future, it would be interesting to investigate whether Kti12 interacts genetically with the same elongation factors that Elongator was shown to interact with (Kong et al., submitted for publication). Finally, it should be noted that according to the data of Fig. 4.8, Kti12 could be cross-linked to DNA in a gene- and Elongator-independent manner.

Though the significance of this result is currently unknown, it might point towards an Elongator-independent role for Kti12 in the cell.

In the last part of Chapter 4, some preliminary experiments concerning the mechanism of action of the zymocin toxin in *S. cerevisiae* cells are presented (Fig. 4.9, 4.10, 4.11). These studies, as well as others, suggest that Elongator and Kti12 are mediators of zymocin's effect in the cell (Fichtner et al., 2002a; Frohloff et al., 2001). In fact they seem to be the main targets of the toxin, working upstream in the pathway of zymocin action. This is the most likely explanation for the finding that the otherwise very sensitive *gcn5Δ* cells become insensitive when either the *ELP3* or the *KTI12* gene is also deleted (Fig. 4.9).

Schaffrath and co-workers previously observed that the presence of zymocin correlates with stalled, un-phosphorylated RNA polymerase II in the early coding region of transcribed genes (Jablonowski and Schaffrath, 2002). Moreover, they reported a reduction in the total cellular amount of that form of the enzyme as a result of zymocin treatment. One interpretation of these results would be that zymocin results in persistently stalled RNA polymerase II, which is then recognized by "recovery" machinery resulting in RNAPII being degraded. This mechanism is reminiscent of the proposed role for Def1 protein during transcript elongation in the presence of DNA damage (Woudstra et al., 2002).

In vivo studies in yeast suggested that Def1 has a role in UV-induced ubiquitination and subsequent degradation of RNA polymerase II. In particular, *def1Δ* mutant cells are un-able to degrade RNA polymerase II in response to UV. With these results in mind, it was obvious to test whether Def1 had any role in zymocin's mechanism of

action. Interestingly, *def1Δ* mutant cells were found to be much more sensitive to the toxin than wild-type cells (Fig. 4.10). The increased sensitivity was shown to correlate with the rate of growth in a culture medium after toxin addition (Fig.4.11). If the role of Def1 in response to zymocin was similar to that proposed for the response to UV-irradiation, RNA polymerase II should become degraded in a Def1-dependent manner in response to zymocin treatment. However, the opposite turned out to be true: in the presence of zymocin the level of RNAPII was indeed dramatically decreased, but RNAPII degradation was much more pronounced in *def1Δ* cells than in wild-type cells (Fig. 4.11). This result suggests that, if the mechanism of zymocin action indeed involves proteolysis of RNA polymerase II, this degradation does not require Def1.

For the future, it needs to be tested whether the reduction of RNA polymerase II level is a direct consequence of RNAPII stalling, or whether it is an indirect effect of another so far unknown mode of zymocin action. Additionally, it seems relevant to study whether zymocin results in ubiquitination of RNA polymerase II, as happens in response to UV. Moreover, the effect of zymocin on cells with other defects in the protein degradation pathways (such as components of the ubiquitination machinery), should be studied genetically and biochemically. Finally, whether zymocin directly results in stalled RNA polymerase II could also be tested by *in vitro* transcription assays, where the effect of purified zymocin and the role of Elongator in its function could be monitored.

In summary, this study has described the molecular architecture of the six-subunit Elongator complex. Moreover, several lines of experimental approaches were pursued to shed light on the

possible roles of the individual Elongator subunits. Finally, a physical and functional connection between Elongator and Kti12 was delineated. In the future, it will be interesting to further dissect the role of Elongator in the transcription cycle and chromatin modification, and to explore the level at which Kti12 is regulating Elongator's activity.

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APPENTIX I

PUBLICATIONS

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RNA Polymerase II Elongator Holoenzyme Is Composed of Two Discrete Subcomplexes*

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Elongator is a histone acetyltransferase complex that associates with the elongating form of RNA polymerase II. We purified Elongator to virtual homogeneity via a rapid three-step procedure based largely on affinity chromatography. The purified factor, holo-Elongator, is a labile six-subunit factor composed of two discrete subcomplexes: one comprised of the previously identified Elp1, Elp2, and Elp3 proteins and another comprised of three novel polypeptides, termed Elp4, Elp5, and Elp6. Disruption of the yeast genes encoding the new Elongator proteins confers phenotypes indistinguishable from those previously described for the other *elp* mutants, and concomitant disruption of genes encoding proteins in either subcomplex does not confer new phenotypes. Taken together, our results indicate that holo-Elongator is a functional entity *in vitro* as well as *in vivo*. Metazoan homologues of Elp1 and Elp3 have previously been reported. We cloned the human homologue of yeast *ELP4* and show that this gene is ubiquitously expressed in human tissues.

The form of RNA polymerase II (RNAPII)[1] responsible for transcript elongation is fundamentally different from the form that enters a promoter to form a preinitiation complex (1, 2). During initiation, RNAPII is hypo-phosphorylated and associated with the functionally conserved Mediator complex, a multisubunit factor required for regulation of transcription (3, 4). The association of RNAPII with Mediator and the general transcription factors is severed during promoter clearance, triggered by TFIIH-mediated hyperphosphorylation of the carboxyl-terminal repeat domain (CTD) of the largest RNAPII subunit (5–7). During elongation, hyperphosphorylated yeast RNAPII is associated with the Elongator complex. Elongator binds directly to RNAPII, at least partly via the CTD, and the interaction is stabilized by CTD hyperphosphorylation (8).

Elongator was biochemically isolated as a component of elongating RNAPII from salt-stable chromatin but can also be isolated as a discrete, three-subunit complex when starting from the DNA-free soluble fraction of a whole cell extract (8). The genes encoding the Elongator subunits have been identified and shown to play a role in transcription elongation *in vivo*: *ELP1* encodes a protein without discernable motifs (8), whereas Elp2 has multiple WD40 repeats (9), and Elp3 has histone acetyltransferase (HAT) motifs (10). The identification of a highly conserved HAT associated with elongating RNAPII suggests a mechanism for modification and remodeling of chromatin during transcript elongation. Recombinant Elp3 indeed has HAT activity in a gel-based HAT assay, and mutations in the sequence encoding the predicted acetyl-CoA binding pocket of the protein significantly reduce this activity (11). Importantly, when the same point mutations are introduced in yeast, they confer phenotypes that are virtually identical to those resulting from deletion of either of the *ELP* genes, indicating that the catalytic activity of the Elp3 protein is essential for Elongator function. The *in vivo* function of Elp3 is overlapping with that of the prototype HAT, Gcn5, the catalytic subunit of SAGA/ADA (12). In the absence of both Elongator and SAGA HAT activity, cells are sick and unable to grow under a large variety of conditions. These phenotypes can be suppressed by concomitantly deleting the genes encoding two histone deacetylases (HDACs), indicating functional redundancy between HDACs as well and supporting the notion that maintenance of a certain overall acetylation level in a cell may be important for cell viability and growth (11). Elongator function, such as HAT activity, is required for normal activation of a number of genes, indicating that Elongator is involved in creating a chromatin structure that is amenable to efficient transcription (8, 10).

To further our understanding of Elongator function, we isolated the complex from extracts of yeast cells in which the gene encoding Elp1 had been modified to express a double affinity-tagged version of the protein. This tag made it possible to rapidly purify Elongator to virtual homogeneity under mild conditions. Here we show that the purified factor is a six-subunit complex, comprised of two discrete, three subunit subcomplexes that easily dissociate. We have identified the three novel proteins of the complex, as well as a human homologue of one of the encoding genes, and provide evidence that holo-Elongator is the functional entity of Elongator *in vivo*.

EXPERIMENTAL PROCEDURES

Expression of Tagged Elp1—A sequence encoding a $(His)_{10}$-HA epitope tag followed by a transcription termination signal was created by polymerase chain reaction using primers 5′-AGCTGACTAGTCATCACCATCACCATCACCATCACCATCACTATCCATGTTCCTGACTATGCCTAATTCCGGGCGAATTTCTTATG-3′ and 5′-GTTCTGAGCTCT-

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[1] The abbreviations used are: RNAPII, RNA polymerase II; CTD, carboxyl-terminal repeat domain; HA, hemagglutinin; FPLC, fast protein liquid chromatography; MALDI-reTOF, matrix-assisted laser desorption/ionization reflectron time-of-flight; NTA, nitrilotriacetic acid; HAT, histone acetyltransferase.

TABLE I
List of yeast strains used

Name	Description	Reference/Source
W303-1A	MATa, leu2-2, 112, his3-11, 15, trp1-1, ade 2-1 can1-100	Thomas and Rothstein (25)
W303-1B	MATα, leu2-2, 112, his3-11, 15, trp1-1, ade 2-1, can1-100	Thomas and Rothstein (25)
JSY100	As W303-1A, but elp1Δ::*LEU2*	Otero et al. (8)
JSY671	As W303-1A, but elp4Δ::*ADE2*	This study
JSY785	As W303-1A, but elp5Δ::*KAN*	This study
JSY537	As W303-1A, but ELP1(His$_{10}$-HA)::*TRP1*	This study
JSY788	As W303-1B, but elp1Δ::*LEU2*	This study
JSY670	As W303-1B, but elp4Δ::*ADE2*	This study
JSY784	As W303-1B, but elp5Δ::*KAN*	This study
JSY786	As W303-1B, but elp6Δ::*HIS3*	This study
JSY787	As W303-1B, but elp1Δ::*LEU2 elp4*Δ::*ADE2*	This study
BJ2168	MATa, prc1-407, prb1-1122, pep4-3, leu2, trp1, ura3-52, gal2	Jones (26)
JSY549	As BJ2168, but ELP1(His$_{10}$-HA)::*TRP1*	This study

TACGCCAAGCTTGCATGCCGGT-3′ and plasmid pAS2-1 (CLONTECH) as a template. The resulting sequence was inserted between the *Spe*I and *Sac*I sites of pRS304 (13) to yield vector pSE.HISHA-304. Part of the *ELP1* open-reading frame was amplified using primers 5′-GCTACACTCGAGACAAGATAATGAGCCTTTACGCCG-3′ and 5′-TGTGACACTAGTAAAATCAACAATATGACTCTTAGGG-3′ and cloned into pSE.HISHA-304 using the *Xho*I and *Spe*I sites yielding plasmid pELP1-HISHA-304. After transformation of the protease-deficient *Saccharomyces cerevisiae* strain BJ2168 (Table I), a TRP$^+$ clone was isolated in which the 3′-end of the *ELP1* gene was replaced, resulting in expression of an Elp1-(His)$_{10}$-HA fusion protein (Elp1-HisHA; strain JSY549). To ensure that the (His)$_{10}$-HA epitope tag did not interfere with Elp1 function, the same integration was performed in the W303 strain background, making it possible to do phenotypic analysis and comparison with wild type and *elp1*Δ cells.

Protein Purification—DNA-free soluble whole cell extract (typically from 0.8–1.0 kg of yeast paste) was prepared from strain JSY549 and subjected to cation-exchange chromatography on Bio-Rex 70 (Bio-Rad) essentially as described previously (8). Protein was stepwise eluted with buffer A (40 mM Hepes-KOH pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, 20% (v/v) glycerol) containing 150 mM, 300 mM, 600 mM, and 1200 mM KOAc. The vast majority of Elongator eluted in the fraction containing 600 mM KOAc. Typically, 50 ml of this fraction was incubated with 0.8 ml of protein A-Sepharose CL4B-12CA5 monoclonal antibody resin (1–3 mg of antibody/ml of resin) overnight at 4 °C. The resin was collected by gravity flow in a column holder and washed extensively with buffer A containing 600 mM KOAc and equilibrated in A containing 300 mM KOAc. Bound proteins were then eluted in three washes with 1 ml of buffer A containing 300 mM KOAc and 2 mg/ml HA peptide (KKKRILKMYPYDVPDYARIL) for 15 min at 30 °C. These fractions were pooled and diluted with an equal volume of buffer (40 mM Hepes-KOH pH 7.6, 300 mM KOAc, 20% (v/v) glycerol) and allowed to bind to 0.4 ml Ni^{2+}-NTA-agarose (Qiagen) at 4 °C overnight with mixing. The resin was collected in a column holder and washed with 2 ml of buffer A containing 300 mM KOAc. After washing with 2 ml of the same buffer containing 300 mM KOAc and 10 mM imidazole, bound protein was eluted in 1 ml of the same buffer containing 300 mM imidazole. Fractions were dialyzed against buffer A containing 100 mM KOAc and stored in small aliquots at −80 °C.

Gel filtration was carried out on a SMART chromatography system (Amersham Pharmacia Biotech). A portion of the eluate from the anti-HA immunoaffinity column was applied via a 25-μl sample loop onto a Superose 6 PC1.6/30 column (Amersham Pharmacia Biotech) collecting 50-μl fractions. Aliquots (1 and 5 μl, respectively) were analyzed by immunoblotting and staining with silver nitrate. Different Elongator (sub)complexes were obtained by anion-exchange chromatography using an ÄKTA-FPLC (Amersham Pharmacia Biotech). Anti-HA eluate fractions 1–3 were pooled (~2 ml), diluted with buffer B (25 mM Tris-HAc, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol) to a final concentration of 150 mM KOAc and loaded onto a Mono Q HR5/5 FPLC column (Amersham Pharmacia Biotech). The column was washed with 2 volumes of buffer B containing 150 mM KOAc and developed with a 10-column volume linear gradient from 150 to 1500 mM KOAc collecting 0.35-ml fractions. Elongator subcomplexes eluted between 1000 and 1450 mM KOAc. Aliquots were analyzed by immunoblotting (1 μl) and staining with silver nitrate (5 μl).

Protein Identification—Gel-fractionated proteins were digested with trypsin, and the mixtures were fractionated on a Poros 50 R2 RP microtip (14). Resulting peptide pools were then analyzed by matrix-assisted laser desorption/ionization reflectron time-of-flight (MALDI-reTOF) MS using a Reflex III instrument (Brüker Franzen; Bremen, Germany) and by electrospray ionization (ESI) MS/MS on an API 300 triple quadrupole instrument (PE-SCIEX; Thornhill, Canada), modified with an ultrafine ionization source (15). Selected mass values from the MALDI-TOF experiments were taken to search a *S. cerevisiae* subset of the protein non-redundant data base (NR; NCBI, Bethesda, MD) using the PeptideSearch (16) algorithm. MS/MS spectra were inspected for y″ ion series to compare with the computer-generated fragment ion series of the predicted tryptic peptides.

Preparation of Antibodies—To produce antibodies recognizing Elp4, Elp5/Iki1, and Elp6, peptides encompassing an amino-terminal cysteine residue followed by the final carboxyl-terminal 19 amino acids of the corresponding predicted open-reading frames were synthesized. Each of these peptides was coupled via its amino-terminal cysteine residue to Keyhole Limpet Hemocyanin (Calbiochem) by *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce) cross-linking and used to immunize rabbits (Murex). For immunoblotting, the anti-Elp4, -Elp5, and -Elp6 antibodies and their respective prebleeds were used at a final dilution of 1:1000.

Yeast Strains and Phenotypic Analysis—All *S. cerevisiae* stains used for genetic analysis (Table I) were congenic with strain W303 and grown and manipulated as described previously (8, 10). To analyze killer toxin sensitivity, yeast strains were transformed with plasmid pNW064 encoding the killer toxin γ-subunit under control of the inducible *Gal1-10* promoter (17). Dilutions of the indicated yeast strains were spotted onto SD (−ura −trp) medium containing 2% glucose or galactose as indicated.

Expression Analysis of Human Elp4—A full-length human Elp4 cDNA clone was obtained from the NEDO sequencing project (clone KAT08960, GenBank™ accession number AK000505). The entire open-reading frame of human Elp4 was amplified by polymerase chain reaction using primers p123 5′-GAAGATCTCCATGGCGGCAGTGGCAACCTG-3′ and p124 5′-GAAGATCTCTAGAAGTCCAGGTGCTTCTTGCC-3′ and radiolabeled with random hexamers. This probe was hybridized to a human multiple tissue Northern blot according to the manufacturer's guidelines (CLONTECH). As a control, the blot was probed with a human β-actin cDNA (CLONTECH).

RESULTS

Purification of Elongator from Soluble Whole Cell Extracts—Previously, we purified the Elongator complex from the DNA-free, soluble fraction of a whole cell extract through five to six conventional chromatography steps (8). To facilitate the purification of Elongator and the subsequent analysis of its composition and enzymatic activities, we constructed a haploid *S. cerevisiae* strain expressing a tagged version of the gene encoding the largest subunit of Elongator, *ELP1*. The endogenous chromosomal copy was replaced with a gene encoding full-length Elp1 fused to a carboxyl-terminal decahistidine stretch and a HA epitope tag (Fig. 1*A*). After verification that the tag did not interfere with Elp1 function *in vivo* (data not shown), we purified Elongator using an efficient three step procedure including two high affinity chromatography steps (Fig. 1*B*). First, soluble whole cell extract was loaded onto Bio-Rex 70 cation-exchange resin. Bound proteins were eluted with buffer containing 300 mM, 600 mM, and 1200 mM potassium acetate (KOAc), respectively. Essentially all Elongator was collected in

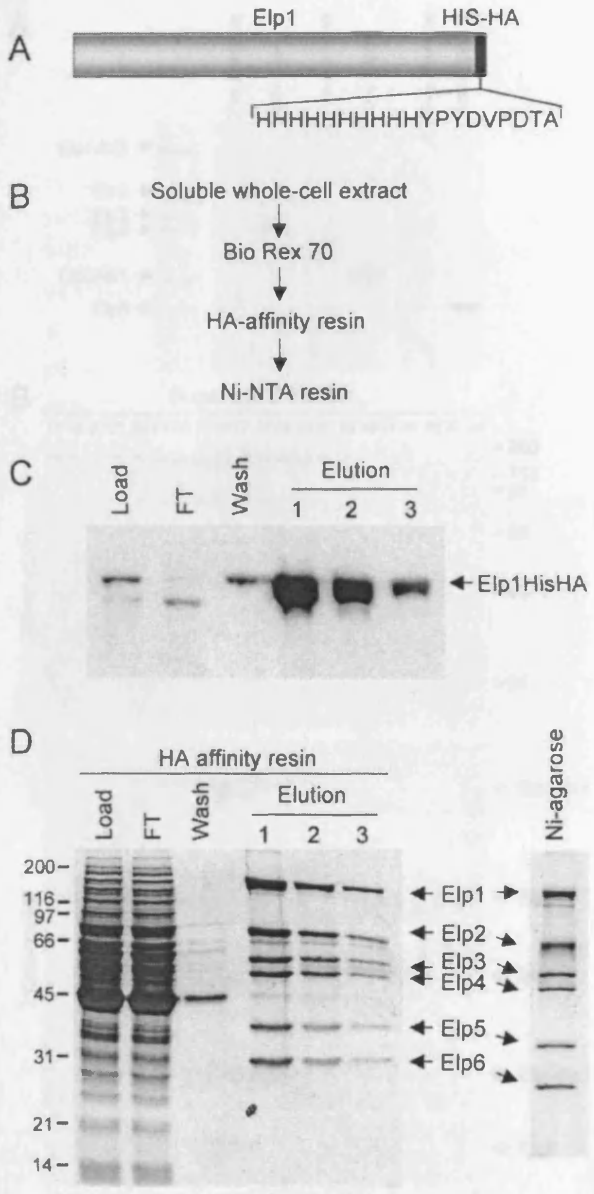

FIG. 1. **Purification of Elongator complex.** *A*, schematic diagram of tagged Elp1 consisting of full-length Elp1 fused to a decahistidine stretch and an HA epitope tag. *B*, diagram of the purification scheme. *C*, immunoblot analysis of fractions from the anti-HA immunoaffinity column. Elp1 protein was detected using rat monoclonal antibody 3F10 (Roche Molecular Biochemicals) recognizing the HA epitope. *D*, protein staining with silver nitrate of Elongator-containing fractions from the anti-HA immunoaffinity resin and the Ni^{2+}-NTA-agarose column. Elp proteins are indicated. Numbers on the *left* indicate the positions of the molecular size markers.

the 600 mM KOAc eluate and subsequently applied onto anti-HA immunoaffinity resin. Analysis by immunoblotting showed that virtually all Elp1-HisHA from the Bio-Rex 70 fraction bound to this column and could be eluted by competition with excess peptide containing the HA epitope (Fig. 1*C*). Interestingly, protein silver staining showed that, in addition to Elp1, Elp2, and Elp3, three additional proteins with apparent molecular weights of 50, 35, and 30 kDa eluted from the immunoaffinity column (Fig. 1*D*). These three proteins did not bind to the immunoaffinity resin in the absence of tagged Elp1 (data not shown), indicating that they interact specifically with Elp1. Moreover, when the eluates from the anti-HA immunoaffinity resin were subjected to Ni^{2+}-agarose affinity chromatography, the three additional proteins also co-eluted with the previously defined Elongator subunits, providing further evidence that the interaction is specific (Fig. 1*D*). We designated these putative novel Elongator subunits Elp4, Elp5, and Elp6, respectively.

Identification of Three Novel Elongator Subunits Elp4, Elp5, and Elp6—Peptide mass fingerprinting using MALDI-reTOF mass spectrometry (14, 16, 18) was used to identify the 50-, 35-, and 30-kDa protein bands. We identified the 50-kDa band as the product of the previously defined open-reading frame YPL101W on chromosome XVI (predicted molecular weight M_r 51.2) and named this gene *ELP4*. The p35/Elp5 protein was identified as the product of open-reading frame YHR187W (chromosome VIII, predicted molecular weight M_r 35.2). Interestingly, this gene was previously identified as the insensitive to killer toxin 1 gene, *IKI1*, which was identified in the same genetic screen as *ELP1*/*IKI3* and whose inactivation renders yeast cells insensitive to pGKL killer toxin (19). Finally, the 30-kDa protein band was found to correspond to the open-reading frame YMR312W on chromosome XIII (predicted molecular weight M_r 30.6), which we termed *ELP6*. The predicted molecular weights of the identified open-reading frames correspond well with the apparent molecular masses in all three cases. Analysis of the three amino acid sequences by data base searching did not reveal any obvious domain structure or homology to proteins with known function.

To verify the identity of the 50-, 35-, and 30-kDa protein bands, polyclonal rabbit antibodies directed against the carboxyl-terminal 19 amino acids of Elp4, Elp5, and Elp6 were generated and tested for reactivity toward purified Elongator by immunoblotting. These antibodies, but not the corresponding preimmune sera, specifically recognized p50/Elp4, p35/Elp5, and p30/Elp6, respectively, in the Elongator preparation, confirming the identification of these proteins as components of the complex (Fig. 2*A*).

To establish that Elp4, Elp5, and Elp6 are *bona fide* subunits of Elongator, the purified complex was analyzed by gel filtration chromatography. All six Elongator proteins exactly co-eluted from this resin as judged by protein silver staining and immunoblot analysis using antibodies directed against Elp1, Elp3, Elp4, Elp5, and Elp6 (Fig. 2*B*). This experiment shows that Elp4, Elp5, and Elp6 are indeed subunits of Elongator and indicates that Elongator is a stoichiometric complex composed of six subunits.

Elongator Is Composed of Two Subcomplexes—Previously, we identified Elongator as a three-subunit complex (8). Careful analysis of protein fractions obtained from these earlier purifications showed that proteins with apparent molecular weights corresponding to Elp4, Elp5, and Elp6 eluted from Mono Q slightly later than Elp1, Elp2, and Elp3 (data not shown). We reasoned that Elongator complex might be disrupted by anion-exchange chromatography on Mono Q, or by high salt concentration. Indeed, when affinity-purified six subunit Elongator was loaded onto Mono Q and eluted with increasing salt, three different forms of the complex could be identified (Fig. 3). First, six subunit holo-Elongator eluted at <1100 mM KOAc. Second, the previously identified form of Elongator composed of Elp1, Elp2, and Elp3 (core Elongator) was detected, and, finally, fractions highly enriched in Elp4, Elp5, and Elp6 were obtained. We also observed that Elongator was disrupted in the presence of 2 M NaCl (data not shown). We conclude that Mono Q chromatography and/or high salt concentration can disrupt the Elongator complex, explaining our failure to previously identify the three smallest subunits. These findings indicate that Elongator is composed of two subcomplexes: one comprising the three largest subunits, and

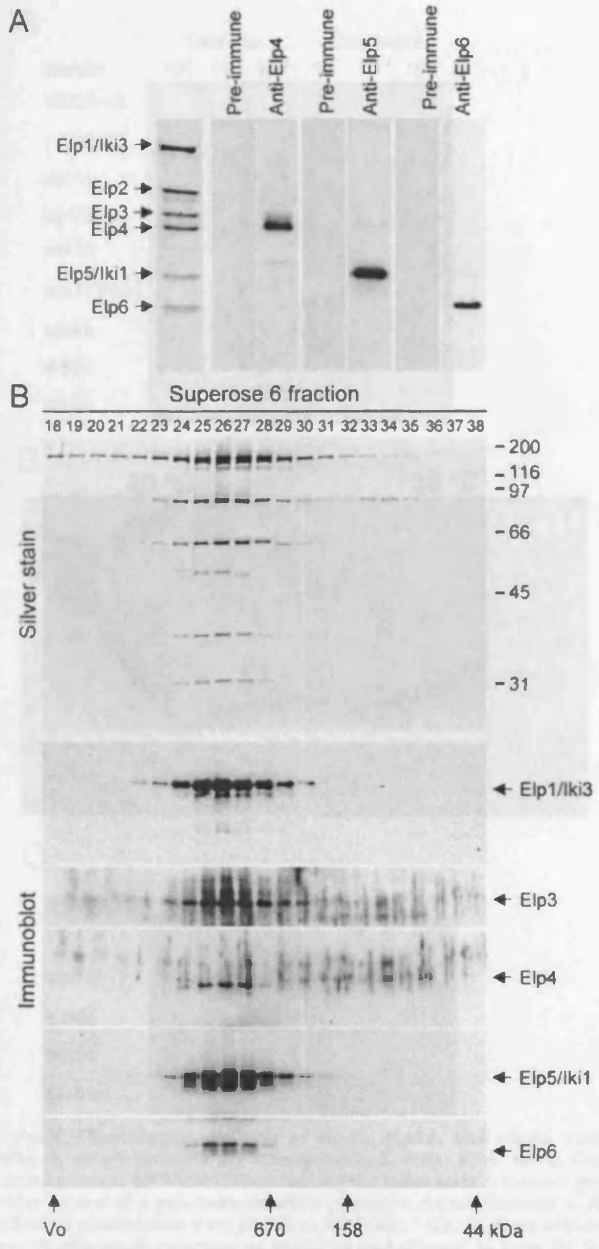

FIG. 2. **Identification of the Elp4, Elp5, and Elp6 proteins by immunoblotting, and analysis of purified Elongator complex by gel filtration.** *A*, protein staining with silver nitrate and immunoblot analysis of purified Elongator using antibodies directed against the carboxyl termini of Elp4, Elp5, and Elp6, respectively. *B*, fractions from the anti-HA immunoaffinity column were subjected to Superose 6 gel filtration chromatography. Fractions were analyzed by 10% SDS-polyacrylamide gel electrophoresis and stained with silver nitrate (*top panel*) or antibodies directed against Elongator proteins (*bottom panels*). Numbers on the *right* of the silver stained gel indicate the positions of the molecular size protein markers. Numbers at the *bottom* of the figure indicate the migration of globular molecular size markers during gel filtration.

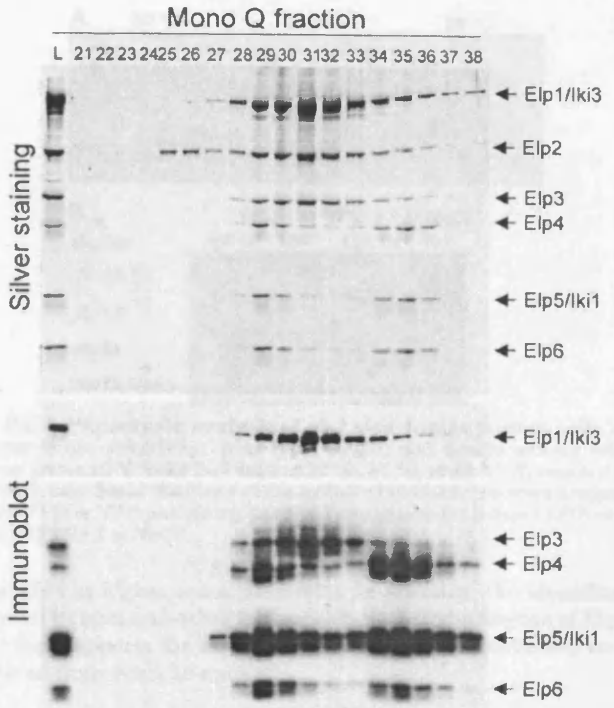

FIG. 3. **Resolution of different forms of Elongator by Mono Q anion-exchange chromatography.** Fractions from the anti-HA immunoaffinity column were loaded onto a Mono Q FPLC column and eluted with a linear salt gradient. Fractions were analyzed by 10% SDS-polyacrylamide gel electrophoresis and stained with silver nitrate (*top panel*) or detected by antibodies directed against Elongator proteins (*bottom panels*).

the second composed of the three newly identified proteins, Elp4, Elp5, and Elp6.

Disruption of ELP4, ELP5, and ELP6 Genes Results in Typical Elp Phenotypes—To analyze the role of *ELP4*, *ELP5*, and *ELP6 in vivo*, we disrupted one copy of the respective entire open-reading frames in diploid yeast cells, which were subsequently induced to sporulate. After dissection of the resulting tetrads, we noted the appearance of small colonies, which occurred in a 2:2 ratio and co-segregated with the marker used (data not shown). This is reminiscent of the phenotype we previously observed in *elp1*Δ, *elp2*Δ, and *elp3*Δ cells (8–10). The identification of two genes encoding Elongator components, *ELP1/IKI3* and *ELP5/IKI1*, as genes whose inactivation cause insensitivity to pGKL killer toxin (19), prompted us to investigate whether disruption of the remaining four *ELP* genes also render cells insensitive to the killer toxin derived from the yeast *Kluyveromyces lactis*. Therefore, we conditionally expressed the γ killer toxin subunit intracellularly using a galactose-inducible promoter (17). As shown in Fig. 4*A*, all *elp*Δ mutant strains grew normally on medium containing glucose where the killer toxin was not expressed. However, upon expression of killer toxin on galactose-containing medium, wild type cells were unable to grow, whereas all *elp*Δ mutants were insensitive to killer toxin. The mechanism of killer toxin depends on the histone acetyltransferase activity of Elongator, as inactivation of the complex in an *elp3* strain carrying a point mutation in a residue important for the catalytic activity of Elp3 also resulted in insensitivity to killer toxin.

Other phenotypes we observed for *elp4*Δ, *elp5*Δ, and *elp6*Δ cells were an inability to grow at 39 °C (Fig. 4*B*) and salt sensitivity (Fig. 4*C*), which were previously observed for *elp1*Δ, *elp2*Δ, and *elp3*Δ mutants (8–10). In addition, *elp1*Δ *elp4*Δ double mutants cells displayed phenotypes virtually identical to those of the single mutants, such as growth rate, and sensitivity to elevated temperature and high salt (Fig. 5 and data not shown), as previously observed for all combinations of *elp1*Δ, *elp2*Δ, *and elp3*Δ mutations (9). Taken together, these results indicate that *ELP1* through *ELP6* are all non-essential genes whose products also form a functional entity *in vivo*.

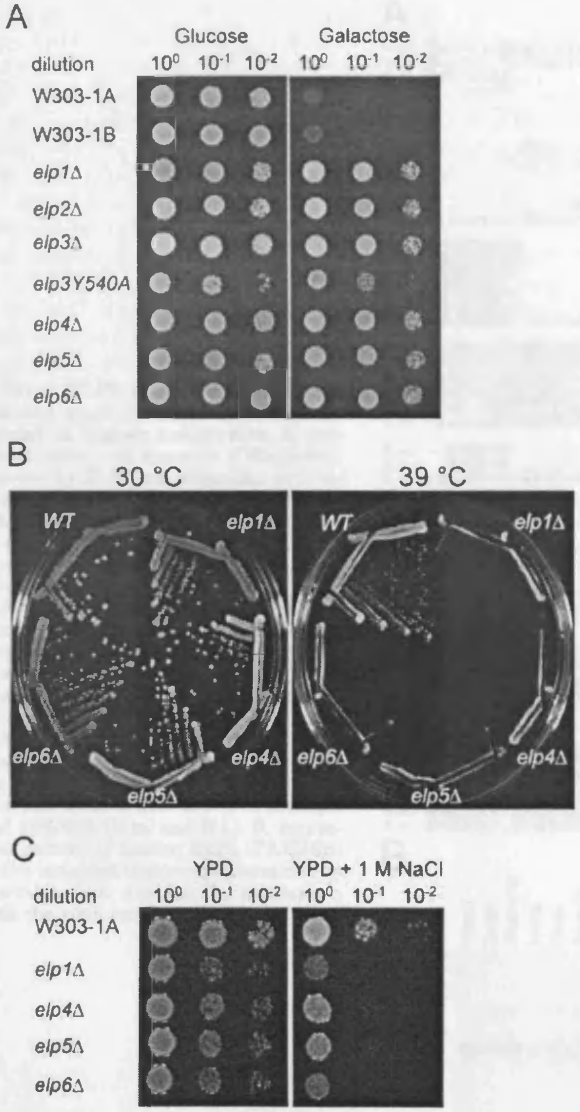

FIG. 4. **Phenotypic analysis of *elp4Δ*, *elp5Δ*, and *elp6Δ* yeast cells.** *A*, all *elp* mutants are insensitive to *K. lactis* killer toxin. Cells carried plasmid pNW064 (17) containing the killer toxin γ subunit gene under control of a galactose-inducible promoter. Serial dilutions of the indicated mutant cells were plated on SD (−ura −trp) medium containing 2% glucose or galactose as indicated and allowed to grow for 2–3 days at 30 °C. *B*, temperature sensitivity. Cells were grown on YPD for 2–4 days at the indicated temperature. *C*, sensitivity to high salt concentration. Serial dilutions of the indicated mutant cells were dropped onto YPD or YPD containing 1 M NaCl and grown for 2–3 days at 30 °C.

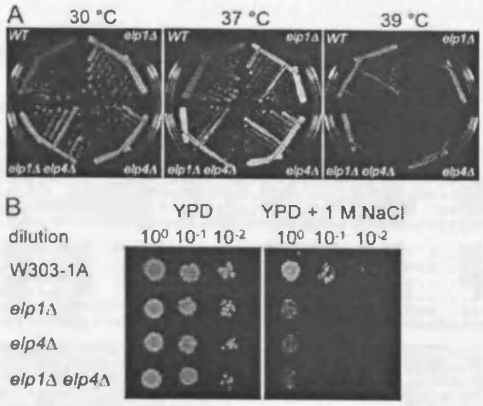

FIG. 5. **Phenotypic analysis of *elp1 elp4* double mutant cells.** *A*, temperature sensitivity. Wild type, single, and double mutant cells were grown on YPD for 2–4 days at 30 °C, 37 °C, or 39 °C. *B*, sensitivity to high salt. Serial dilutions of the indicated mutant cells were dropped onto YPD or YPD containing 1 M NaCl and grown for 2 days (*YPD*) or 3 days (*YPD+1 M NaCl*).

Identification of Elp4 Homologues in Higher Eukaryotes—Further data base searching using the predicted amino acid sequence of *ELP4* as a query identified significant homology with several open-reading frames from a variety of higher eukaryotes, including human and mouse (Fig. 6*A*). No clear domain structure could be identified on the basis of these homologies. Interestingly, however, the putative human and mouse Elp4 proteins are encoded by the human and mouse *PAXNEB* gene, respectively, which is localized on human chromosome 11p13 (mouse chromosome 2), a region implicated with human disease (20). *PAXNEB* is expressed in a variety of human tissues as determined by Northern blot analysis (Fig. 6*B*) and indicated by the presence of multiple expressed sequence tags (ESTs) derived from different tissues in the data base. The ubiquitous expression pattern suggests a general role for Elp4 in higher eukaryotic cells. In addition, the identification of human and other higher eukaryotic homologues of Elp4 further supports the notion that Elongator is structurally conserved from yeast to man.

DISCUSSION

In the present study we isolated Elongator complex by utilizing a rapid purification procedure based largely on affinity chromatography. Our findings can be summarized as follows: Elongator consists of six subunits; the three identified previously, and a novel discrete subcomplex composed of three proteins, termed Elp4, Elp5, and Elp6. We have named the novel factor holo-Elongator, to distinguish it from the core-factor (Elp1, Elp2, and Elp3) that we isolated previously. holo-Elongator is a labile complex that can be dissociated into its two three-subunit subcomplexes by treatment with high salt or by anionic chromatography. We have identified the genes encoding the three new subunits and shown by genetic analysis that strains lacking any one of these genes have phenotypes that are indistinguishable from those of the previously characterized *elp* strains. Finally, we have cloned the human homologue of yeast *ELP4* and shown that it is ubiquitously transcribed.

holo-Elongator Is a Functional Unit—In vivo, two of the proteins in the Elp4/Elp5/Elp6 module, Elp5 and Elp6 have been shown to interact in a large scale two-hybrid screen (21). We found that deletion of any gene encoding a component of holo-Elongator results in similar phenotypes and that concomitant deletion of more *ELP* genes fails to confer a new phenotype. In this respect, Elongator seems fundamentally different from other transcription-related multisubunit complexes, such as Mediator, Swi/Snf, Rsc, ADA, and SAGA. Mutation of genes encoding subunits of these complexes often confer very different phenotypes, indicating that these factors are at least partly composed of subcomplexes or components with different specialized (gene-specific) functions. Additionally, some of the components of these factors, such as Gcn5 (a component of both ADA and SAGA) and Arp7/9 (components of both Swi/Snf and Rsc) perform functions in more than one factor, which likely contributes to diversified phenotypes (12, 22). Based on these observations and the above-mentioned fundamental difference between Elongator and other multisubunit factors, it may be argued that the products of the *ELP* genes are likely to only exert their function together, in the context of Elongator. In agreement with this, we have failed to observe other proteins than the six holo-Elongator subunits after affinity purification from a strain expressing epitope-tagged Elp4 (TP and JQS,

FIG. 6. **ELP4 is a ubiquitously expressed gene whose product is conserved in higher eukaryotes.** *A*, predicted amino acid sequence of the protein encoded by *ELP4* and alignment with sequences from various species using ClustalX (24). *M.m.*, *Mus musculus*, Q9ER73; *H.s.*, *Homo sapiens*, Q9NX11; *D.m.*, *Drosophila melanogaster*, Q9VMQ7; *C.e.*, *Caenorhabditis elegans*, Q18195; *S.p.*, *Schizosaccharomyces pombe*, Q9USP1; *S.c.*, *S. cerevisiae*. Conserved residues are shaded in *light gray*, identical residues in *dark gray*, and residues identical in all species are *highlighted*. Yeast Elp4 is 26% identical/41% similar to human Elp4 (PAXNEB) over the entire sequence. Otherwise, homology between these proteins range from 21% identical and 41% similar (S.c. and D.m.) to 26%/43% (S.c. and S.p.) and 30%/49% (D.m. and H.s.). *B*, expression pattern of human ELP4 (PAXNEB) in the indicated tissues as determined by Northern blot analysis in comparison with the actin control.

data not shown). It may also be significant that a point mutation resulting in loss of Elp3 HAT activity also confers the full *elp* phenotype. This indicates that the most important function of holo-Elongator lies in its capacity as a HAT.

Elongator As a Putative Target for K. lactis Killer Toxin—ELP1 is identical to *IKI3,* and the newly identified *ELP5* gene is identical to *IKI1.* We found that the interesting and intriguing insensitivity to expression of the *K. lactis* killer γ-toxin is a phenotype shared by all the *elp* strains. While this work was in progress, Frohloff *et al.*(23) reported the identification of mutants from a killer toxin screen virtually identical to the *IKI* screen and named their isolated mutants *TOT* (for toxin target). From this screen, *TOT1*, *2*, and *3* were found to be identical to *ELP1*, *ELP2*, and *ELP3*, respectively. The authors also found that the protein product of *IKI1/TOT5,* as well as the product of a gene isolated in another killer toxin screen, *KTI12/TOT4* (killer toxin insensitive 12*)*, could be co-immunoprecipitated with Elongator proteins, indicating that all the gene products isolated so far as intracellular effectors of the killer toxin interact. Our identification of Iki1/Tot5 as a component of holo-Elongator (Elp5) provides an explanation for the Iki1/Tot5-Elongator interaction observed by Frohloff *et al.* (23). By contrast, extensive analysis by mass spectrometry did not provide any evidence for the presence of Kti12 in highly purified Elongator preparations. Because the *kti12/tot4* mutant was shown to have phenotypes strikingly similar to those of *elp* mutants (23), however, it is likely that this protein plays a role in Elongator function. Importantly, expression of *KTI12/TOT4* from multicopy plasmids, but not similar overexpression of *ELP1/TOT1*, *ELP2/TOT2*, *ELP3/TOT3*, or *ELP5/TOT5,* confers γ-toxin resistance (23). Taken together, these findings thus suggest that Kti12/Tot4 is not a component of Elongator, but rather influences its activity. This possibility is presently under investigation.

Elongator Is Conserved among Eukaryotes—Human homologues of *ELP1* and *ELP3* have been identified by searching the data bases (8, 10). In support of the notion that the structure and function of holo-Elongator is highly conserved among eukaryotes, we identified a human homologue of the yeast *ELP4* gene, which has previously been submitted to the data bases and named *PAXNEB*. This gene is ubiquitously expressed, and is located in a region on chromosome 11 that has been implicated in human disease. Heterozygous deletion of the 11p13 region gives rise to *WAGR* syndrome: Wilm's tumor, Aniridia, Genitourinary abnormalities, and mental Retardation. Most of these abnormalities are due to deletion of the well studied disease genes, *PAX6* and *WT1*, but the cause(s) of the mental retardation remains to be identified. Other disease-related loci, such as those associated with loss of heterozygosity in breast and bladder cancers, also map to this region (Ref. 20 and references therein). We are presently isolating human Elongator with the aim to explore the molecular structure and function of Elongator in metazoans.

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Molecular Architecture, Structure-Function Relationship, and Importance of the Elp3 Subunit for the RNA Binding of Holo-Elongator*

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The molecular architecture of six-subunit yeast holo-Elongator complex was investigated by the use of immunoprecipitation, two-hybrid interaction mapping, and *in vitro* studies of binary interactions between individual subunits. Surprisingly, Elp2 is dispensable for the integrity of the holo-Elongator complex, and a purified five-subunit *elp2Δ* Elongator complex retains histone acetyltransferase activity *in vitro*. These results indicate that the WD40 repeats in Elp2 are required neither for subunit-subunit interactions within Elongator nor for Elongator interaction with histones during catalysis. Elp2 and Elp4 were largely dispensable for the association of Elongator with nascent RNA transcript *in vivo*. In contrast, Elongator-RNA interaction requires the Elp3 protein. Together, these data shed light on the structure-function relationship of the Elongator complex.

The Elongator complex was first identified as a component of a hyperphosphorylated RNA polymerase II (RNAPII)[1] holoenzyme isolated from yeast chromatin (1). One of the subunits of Elongator, Elp3, contains domains conserved among histone acetyltransferases (HATs) (2), and Elongator has HAT activity directed against histone H3 and to a lesser extent H4 *in vitro* (3), raising the possibility that Elongator acetylates histones during RNAPII transcript elongation.

Elp3 HAT activity is essential for Elongator function *in vivo* as point mutations that abolish the catalytic function of Elp3 *in vitro* also confer the full range of *elp* phenotypes (4). *elp* phenotypes include temperature sensitivity at 39 °C, salt sensitivity, and slow adaptation to growth on carbon sources such as galactose (1, 5). *elp* cells are also sensitive to caffeine, Calcofluor White, and 6-azauracil (6, 7). Significantly, in all cases tested, *elp* phenotypes correlate with a failure of the mutant cells to activate, in a timely manner, the genes required for growth under the new conditions (1).

An *elp* deletion in combination with the deletion of different genes encoding transcription factors confers synthetic phenotypes. Thus, *elp3* in combination with *rpb9* (encoding an RNAPII subunit) is lethal (8), as is the combination of an *elp3* and *ctk1* mutation (*CTK1* encodes a subunit of the CTD kinase, CTDK1) (9). Cells expressing a conditional allele of *SPT16* (encoding the largest subunit of yeast FACT-CP complex) also display a synthetic phenotype in combination with *elp3* mutation (10). Recent data have shown that Elongator also genetically interacts with Mediator, Rad6 ubiquitin ligase, Paf1 complex, and Rpd3-Sin3 complex.[2] Finally, combining the mutation of *elp3* with *gcn5* (encoding the catalytic subunit of the SAGA-ADA HAT complexes) confers severe growth defects, which are not seen in either of the single mutants (4). Significantly, the *gcn5 elp3* double mutant has reduced levels of histone H3 acetylation in several genes compared with the single mutants, and low levels of acetylation in the coding region of these genes correlates with reductions in gene transcription. By contrast, low levels of acetylation in the promoter of genes does not correlate with reduced transcription nor with reduced promoter occupancy by the TATA-binding protein (11). Recent experiments using RNA-immunoprecipitation have shown that Elongator is indeed present in the coding region of active genes *in vivo* (12).

The Elongator complex was first thought to consist of three subunits, named Elp1, Elp2, and Elp3 (now called core Elongator) (1), but later purification of the complex from a yeast strain expressing epitope-tagged Elp1 has shown that the active complex, holo-Elongator, consists of six subunits (Elp1–Elp6) (13–15), which are organized in two three-subunit subcomplexes (13, 15). Significantly, deletion of any one of the (*ELP*) genes encoding these six subunits confers more or less identical phenotypes, and new phenotypes are not detected upon concomitant deletion of two or three *ELP* genes (5, 13). These data suggest a tight functional connection between the proteins comprising the Elongator complex. Consistent with this idea, holo-Elongator, but not core Elongator, has HAT activity even though both complexes contain the catalytic Elp3 subunit (13).

Here we investigate the molecular architecture of Elongator complex and show that the only *ELP* gene that can be deleted without significant loss of Elongator integrity is *ELP2*. Surprisingly, an Elongator complex lacking Elp2 even retains the ability to acetylate histones *in vitro*. We also show that the association of Elongator with nascent RNA *in vivo* requires Elp3 but not Elp2 or Elp4.

EXPERIMENTAL PROCEDURES

Yeast Strains and Phenotypic Analysis—All *Saccharomyces cerevisiae* stains used for genetic analysis (Table I) were congenic with strain W303 and were grown and manipulated as described previously (1, 4).

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[1] The abbreviations used are: RNAPII, RNA polymerase II; HAT, histone acetyltransferase; HA, hemagglutinin; GST, glutathione *S*-transferase.

[2] S. E. Kong, M. S. Kobor, N. J. Krogan, J. F. Greenblatt, and J. Q. Svejstrup, unpublished results.

TABLE I
Yeast strains

Strain	Genotype	Reference/source
W303-1B	*MATα*	Thomas and Rothstein (23)
JSY 100	*MATα elp1Δ::LEU2*	Otero *et al.* (1)
JSY 118	*MATα elp2Δ::LEU2*	This study
JSY130	*MATα elp3Δ::LEU2*	Wittschieben *et al.* (2)
JSY672	*MATα elp4Δ::ADE2*	Winkler *et al.* (13)
JSY784	*MATα elp5Δ::KAN*	Winkler *et al.* (13)
JSY786	*MATα elp6Δ::HIS3*	Winkler *et al.* (13)
JSY489	*MATα ELP1(His$_{10}$-HA)::TRP1*	Winkler *et al.* (13)
JSY666	*MATα ELP4(His$_{10}$-HA)::TRP1*	This study
JSY662	*MATα elp2Δ::LEU2 ELP1(His$_{10}$-HA)::TRP1*	This study
JSY964	*MATα elp3Δ::LEU2 ELP1(His$_{10}$-HA)::TRP1*	This study
JSY965	*MATα elp4Δ::ADE2 ELP1(His$_{10}$-HA)::TRP1*	This study
JSY973	*MATα elp5Δ::KAN ELP4(His$_{10}$-HA)::TRP1*	This study
JSY974	*MATα elp6Δ::HIS3 ELP4(His$_{10}$-HA)::TRP1*	This study
JSY754	*MATα ELP1(MYC$_{18}$)::HIS3*	This study
JSY969	*MATα elp3Δ::LEU2 ELP1(MYC$_{18}$)::HIS3*	This study
JSY777	*MATαELP3(MYC$_{18}$)::HIS3*	Gilbert *et al.* (12)
JSY968	*MATα elp2Δ::LEU2 ELP3(MYC$_{18}$)::HIS3*	This study
JSY975	*MATα elp4Δ::ADE2 ELP3(MYC$_{18}$)::HIS3*	This study

Expression of Tagged Proteins in Vivo—For the construction of the Elp4-HisHA strain, part of the ELP4 open reading frame was amplified using primers 5′-GCCGGGGTACCGGGGCTGCCGCTGGGAAACTCT-G-3′ and 5′-CCGGCCGGATCCATAGTCTAAAGATATCTTGGTCT-3′ and cloned into pSE.HISHA-304 (16) using the KpnI and BamHI sites to produce plasmid pELP4-HISHA-304. After yeast transformation, a TRP$^+$ clone was isolated in which the 3′-end of the *ELP4* gene was replaced, resulting in expression of an Elp4-(His)$_{10}$-HA fusion protein. To ensure that the (His)$_{10}$-HA epitope tag did not interfere with Elp4 function, phenotypic analysis was performed.

Protein Purification—The procedure for purification of Elongator from the *elp2Δ Elp1-HISHA* strain has been described elsewhere (12).

Yeast Whole Cell Extract Preparation and Western Blot Analysis—Whole cell extracts were prepared as was described elsewhere (17) from the indicated strains. The antibody against α-tubulin was a mouse monoclonal from Oncogene and was used in a final dilution of 1/100 for immunoblotting. The antibodies against Elp1, 2, 3, 4, 5, and 6 were used as described previously (1, 2, 5, 13).

RNA Preparation—For the detection of ELP3 mRNA, total RNA was isolated from an equal number of *S. cerevisiae* cells from the indicated strains using the RNAeasy mini kit (Qiagen) according to the manufacturer's recommendations. The probe was a PCR product from the coding region of the *ELP3* gene. For labeling, Ready-to-Go DNA labeling beads from Amersham Biosciences were used according to the manufacturer's recommendations. Hybridization of the membrane with the probe was performed using the ExpressHyb kit from Clontech. For the detection of 28 and 18 S RNA, the gel was stained with EtBr in a final dilution of 1/10,000 in water.

Co-immunoprecipitation Experiments—For Holo-Elongator co-immunoprecipitation experiments, proteins from the first Bio-Rex chromatography step were used (13). 500 μg of protein adjusted to 500 mM salt in buffer A (12) was incubated with Sepharose A beads, which had been previously conjugated with 12CA5 antibody. The beads were washed three times with the same buffer and resuspended in 1× SDS loading buffer, and the bound proteins were subjected to SDS-PAGE.

Two-hybrid Interactions—The coding sequence of all of the genes encoding the Elongator proteins were cloned in both "GAL4-activation domain"- and "GAL4-binding domain"-based vectors provided from the GAL4 two-hybrid phagemid vector kit (Stratagene). To detect interactions, the plasmids were introduced in genetically modified yeast cells provided from the Matchmaker Library Construction & Screening Kit (Clontech). The interaction studies were done according to the manufacturer's recommendations.

Expression of Recombinant GST and GST-Elp5 in Bacteria—To generate a GST-Elp5 fusion protein, the open reading frame of the *ELP5* gene was cloned in the pGEX-3X vector (Amersham Biosciences), and the plasmid was introduced in BL21 DE3 competent bacteria. Cells were grown at 37 °C and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h. After cell resuspension in phosphate-buffered saline, the cell suspension was incubated for 30 min on ice with lysozyme (50 mg/ml) and then subjected to sonication. The extract was incubated for 1 h at 4 °C with Triton X-100 in a final concentration of 1%, and the soluble supernatant was collected after a 10-min 12,000 rpm spin at 4 °C. This soluble supernatant contained GST-Elp5, which was purified and immobilized on glutathione beads (Amersham Biosciences) by incubation for 30 min at room temperature. The soluble GST control protein was produced in the same way.

In Vitro Transcribed/Translated Proteins—The recombinant Elp4 and Elp6 proteins used for the *in vitro* pull-down experiments were produced using the TnT T7-coupled wheat germ extract systems (Promega) according to the manufacturer's recommendations. The DNA template used was pBluescript II KS (±), into which the coding sequence of the two genes was cloned. Details are available on request.

In Vitro Pull-down Experiments—Immobilized baculovirus-expressed histidine-tagged Elp1 or Elp2 and bacterially expressed GST or GST-Elp5 proteins were mixed with the product of *in vitro* transcription/translation (Elp4 or Elp6) reactions in a buffer containing 250 mM potassium acetate, 100 mM Hepes-KOH, pH 7.6, 20% glycerol, 0.1% Nonidet P-40, 1× proteinase inhibitors, and 4 mM β-mercaptoethanol and incubated overnight. The beads were washed three times before the bound proteins were separated by PAGE on a 10% SDS gel. The Elp4 and Elp6 proteins showed weak nonspecific binding to nickel-agarose. Therefore, these beads were washed three times with the above buffer but containing 30 mM imidazole.

Other Assays—Histone acetyltransferase reactions (15 μl) were carried out as described (3). RNA immunoprecipitation experiments were carried out as described by Gilbert *et al.* (12).

RESULTS

In a multisubunit protein complex, the stability of an individual subunit can be dependent on the presence or integrity of the other subunits. We examined the amount of individual Elongator subunits present in extracts derived from different *elp* deletion strains. Fig. 1*A* (*top panels*) shows the results of an analysis where whole cell extracts were immunoblotted and probed with anti-Elp3 antibodies. As a loading control, reactivity with an anti-tubulin antibody was used. Interestingly, the Elp3 protein either could not be detected or was present at very low levels in cells lacking the *ELP1* gene. In cells lacking any of the other *ELP* genes, the Elp3 levels were also somewhat reduced, but the protein was still detectable (Fig. 1*A*, *top panel*, compare the tubulin/Elp3 ratio in wild type (*WT*) cells with that in the mutants). By contrast, Elp4, Elp5, and Elp6 were only absent from extracts derived from strains in which their encoding gene was deleted (Fig. 1*A*, *middle* and *bottom panels*). Importantly, Northern blot analysis of RNA from the same cells showed that the mRNA level of the *ELP3* gene was comparable in wild type and all of the *elp*Δ cells (Fig. 1*B*) suggesting that reduced protein stability rather than gene expression was causing the effect. We conclude that deletion of the *ELP1* results in substantial destabilization of the Elp3 protein, suggesting that the Elp1 protein is required for the integrity of Elp3, in all likelihood because of a direct interaction between these proteins.

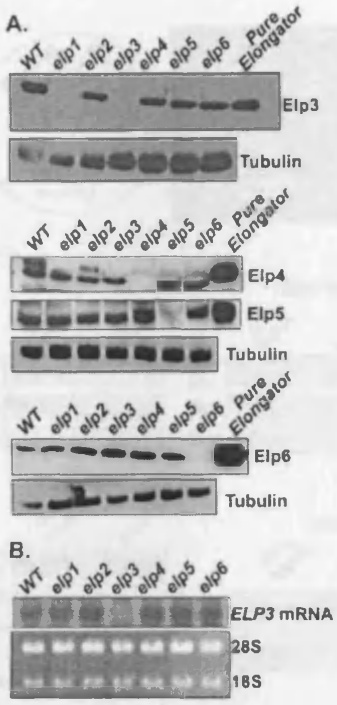

FIG. 1. **Stability and expression of Elongator subunits.** *A*, the presence of Elp3 (*top panels*), Elp4 and Elp5 (*middle panels*), and Elp6 (*bottom panels*) in yeast cells lacking the indicated *ELP* genes was examined by Western blotting using anti-Elp3 antibodies. Detection of tubulin was used as a loading control. *B*, expression of the *ELP3* gene in the indicated *elp* mutants was examined by Northern blotting. Note that the relevant Elp4 band in different cell types is the *lower one*. The reduced mobility of the Elp4 protein in the control lane with pure Elongator is due to Elp4 being tagged (HA-His).

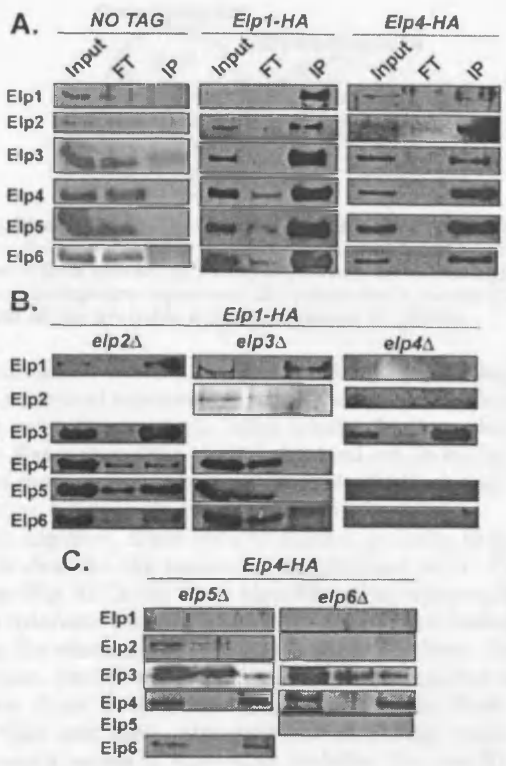

FIG. 2. **Elongator subunit interactions investigated by co-immunoprecipitation.** Immunoprecipitation of a tagged subunit using 12CA5 monoclonal antibodies and Western blotting with antibodies directed against the six Elongator subunits were used to investigate the integrity of the Elongator complex in different mutant strains. *A*, precipitations from wild type strains carrying either no tag, Elp1-HA, or Elp4-HA as indicated. *B*, Elp1-precipitations from the indicated mutant strains. *C*, Elp4 precipitations from the indicated mutant strains.

Protein-Protein Interactions among Elongator Subunits—To investigate protein-protein interactions in the Elongator complex, we next performed co-immunoprecipitation experiments in different *elp* deletion strains. For these experiments whole cell extracts were prepared from cells expressing a tagged version of an Elongator subunit while lacking another one, for example from *elp2* cells expressing Elp1-HA. To enable high efficiency immunoprecipitation and to increase specificity, whole cell extracts were first subjected to chromatography on Bio-Rex before being loaded onto 12CA5-conjugated Sepharose A beads. As shown in Fig. 2*A*, Elongator could, under those conditions, be specifically immunoprecipitated from wild type cell extracts carrying either Elp1 or Elp4 tagged with an HA epitope but not from control cells that did not express a tagged Elp protein. Remarkably, when the *ELP2* gene was deleted, Elp1 still interacted with the five remaining subunits, although the three smaller proteins Elp4, Elp5, and Elp6 appeared to be somewhat substoichiometric (Fig. 2*B*, *left panel*). This experiment indicates that the Elp2 protein is not required for the integrity of the Elongator complex.

When extracts from cells lacking the *ELP3* gene were used, only the Elp1 protein was detected in the precipitates, which indicates that none of the remaining four subunits interact strongly with Elp1 (Fig. 2*B*, *middle panel*). We conclude that the Elp3 protein is not only essential for catalytic function but also for the integrity of the Elongator complex.

In the absence of Elp4, the Elp1 protein could still interact with Elp3 (Fig. 2*B*, *right panel*). However, in this case the Elp2, Elp5, and Elp6 proteins could not be detected even in the inputs. This can be explained in two ways: either those proteins no longer co-elute with Elp1 and Elp3 from Bio-Rex if Elp4 is absent, or deletion of the *ELP4* gene results in reduced Elp2, Elp5, and Elp6 protein levels. Our previous experiments showed that Elp5 and Elp6 protein levels were not decreased significantly in any of the *elp* mutants (Fig. 1*A*), pointing to a lack of interaction with core Elongator as the likely explanation. Unfortunately, the Elp2 protein could not be detected in this case in the Bio-Rex-70 eluate (and could generally not be detected in crude whole cell extracts with our polyclonal anti-Elp2 antibody), precluding any conclusions on the fate of this protein in *elp4* extracts. We conclude that Elp4 is required for the association of Elp2 and the small Elp4/5/6 complex with Elp1 and Elp3.

When extracts from *elp5* cells expressing Elp4-HA protein were used, Elp4 only co-immunoprecipitated Elp6 and low amounts of Elp3 (Fig. 2*C*, *left panel*). This suggests that Elp4 interacts directly with Elp6 and that neither of these two proteins interacts strongly with any of the three larger proteins Elp1, Elp2, and Elp3, although a weak Elp4/Elp6-Elp3 interaction was evident. The absence of a signal for Elp1, even in the input, again precluded firm conclusions on the fate of this protein in *elp5* cells. However, it was obvious from the previous experiment with *elp3*Δ cells that Elp1 does not interact directly with Elp4 or Elp6 (Fig. 2*B*, *middle panel*). In *elp6* cells expressing Elp4-HA protein, Elp4 also only co-precipitated a small amount of Elp3 (Fig. 2*C*, *right panel*), supporting the notion that these proteins interact directly, albeit weakly. Elp4 did not interact with Elp5 in the absence of Elp6. As Elp5 is stable in *elp6* cells (Fig. 1*A*, *middle panels*), this indicates that it is incorporated into the small subcomplex in an Elp6-dependent manner.

We conclude from the above experiments that there is a direct interaction between Elp1 and Elp3, as well as between Elp4 and Elp6. Because a core-Elongator complex consisting of

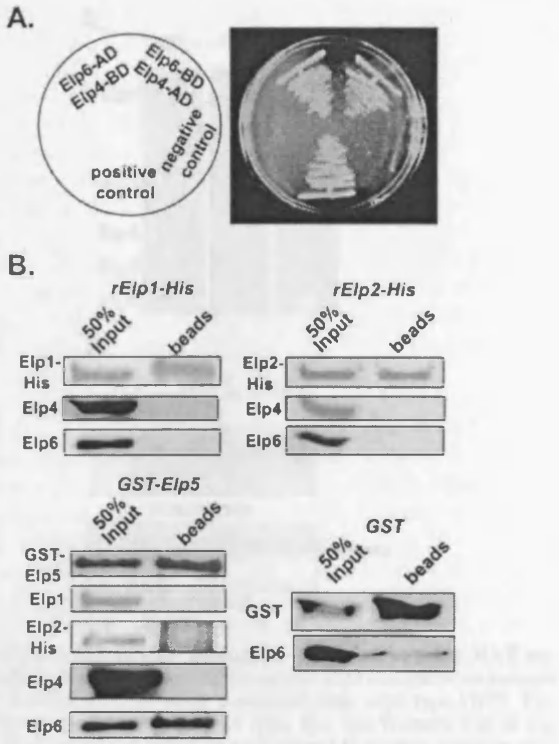

FIG. 3. **Binary Elongator subunit interactions revealed by two-hybrid interaction or direct protein-protein interaction mapping *in vitro*.** *A*, Elp4 and Elp6 interaction shown by two-hybrid interaction. Positive and negative controls were from the Clontech matchmaker kit. Please note that for the large number of negative interaction results we obtained, it was ensured that the relevant Elp-Gal fusion protein rescued the mutant phenotype of the corresponding *elp* strain, showing that the Elp protein in question was correctly folded and functional even in the presence of the Gal moiety. *B*, protein-protein interactions investigated with recombinant Elongator subunits. *Upper left panel*, Elp1 does not directly interact with Elp4 and Elp6. *Upper right panel*, Elp2 does not directly interact with Elp4 and Elp6. *Lower panels*, Elp5 interacts directly with Elp6.

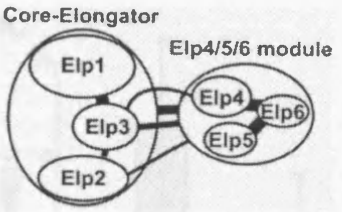

FIG. 4. **Model for the architecture of the yeast holo-Elongator complex.** Spheres delineate the two three-subunit subcomplexes. The thickness of lines between proteins, between protein subcomplexes, and between subcomplexes represents the strength of a particular interaction based on the available evidence. See text for details.

Elp1, Elp2, and Elp3 has previously been isolated (1), the absence Elp2 in Elp1-precipitates from *elp3* cells also indicate that interactions with Elp3 are required for the incorporation of Elp2 into core-Elongator. Surprisingly, the co-immunoprecipitation experiments do not indicate any strong direct binary interactions between any individual protein in the Elp4/5/6 module and any one of the proteins in core Elongator. This suggests that it is primarily novel interaction surfaces created by the association of Elp1, Elp2, and Elp3 in core-Elongator, and Elp4, Elp5, and Elp6 in the small subcomplex, respectively, that are important for the later association of these subassemblies into holo-Elongator.

Pairwise Elongator Protein Interactions—To compliment the co-immunoprecipitation studies, pairwise interactions between individual Elongator proteins were examined by use of the yeast two-hybrid system. The genes encoding all the Elongator subunits were cloned in-frame with the activation domain and the DNA-binding domains of the GAL4 activator, respectively, and tester yeast strains were transformed with combinations of these 12 plasmids. All possible combinations of pairwise Elongator proteins interactions were tested in both directions, but, surprisingly, the only interaction detected was between Elp4 and Elp6 (Fig. 3*A*, negative results not shown).

Finally, we sought to investigate the *in vitro* pairwise interactions of several Elongator components (Fig. 3*B*). Purified recombinant His-tagged Elp1 and His-tagged Elp2 proteins were obtained via expression in insect cells and recombinant GST-Elp5 via expression in bacteria, and Elp4 and Elp6 were transcribed/translated *in vitro*. Unfortunately, despite attempts in several expression systems, we were unable to obtain soluble recombinant or *in vitro* transcribed/translated Elp3 protein. Examples of the results obtained are shown in Fig. 3*B*. Of all of the many combinations that were tested, only a direct interaction between Elp5 and Elp6 was detected.

Taken together, these results make it possible to propose a model to describe the molecular architecture of the Elongator complex (Fig. 4). In the large core-Elongator subcomplex, Elp1 protein interacts with Elp3 but does not interact strongly with Elp2 in the absence of Elp3. This suggests a direct Elp2-Elp3 interaction. On the other hand, the small subcomplex is formed based on direct interactions between Elp4 and Elp6 and between Elp6 and Elp5. Although not absolutely required, the Elp2 protein serves to somewhat stabilize the core Elongator-Elp4/5/6 interaction, whereas Elp3 is essential for the integrity of the complex. Furthermore, a weak interaction between Elp3 and Elp4 was detected. Based on these results we suggest that, rather than relying solely on strong binary interactions between individual subunits of the respective subcomplexes, the formation of the small subcomplex creates new interaction surfaces to enable contacts with Elp3 and Elp2, as well as with novel interaction surfaces created by the association of Elp1, Elp2, and Elp3 into core-Elongator (Fig. 4).

The WD40 Repeat Protein, Elp2, Is Dispensable for the in Vitro HAT Activity of Elongator—The data presented above revealed that although an *elp2* strain displayed the typical Elongator phenotypes (5), the remaining five subunits still exist as a complex in these cells. To investigate whether the five-subunit *elp2*Δ complex also retained Elongator activity, we purified the "mutant" complex and tested its *in vitro* HAT activity. This was particularly relevant, because several HAT and histone deacetylase complexes harbor subunits, which, like Elp2, contain WD40 repeats. It has thus been suggested that WD40 repeats might be involved in contacting histones to facilitate the acetyltransferase and deacetylation reactions, respectively (18, 19). The purification scheme employed was identical to that used previously for the purification of a six-subunit Elongator complex from cells carrying a double affinity-tagged Elp1 protein (13). As indicated by Fig. 5*A*, a purified *elp2*Δ Elongator complex from two independent purifications retained the five other subunits, although the three smaller subunits as well as the Elp3 protein appeared to be somewhat substoichiometric compared with the wild type complex. To be able to compare the HAT activity of the *elp2*Δ complex with that purified from wild type cells, we therefore used similar amounts of the catalytic Elp3 subunit in the HAT reactions, with the consequence that other subunits, particularly Elp1, were present in larger amounts in the reactions containing the mutant five-subunit complex. Fig. 5*B* shows the result of HAT assays comparing wild type and the two independently purified mutant complexes. Surprisingly, Elongator retained *in vitro* HAT activity directed against H3 even in the absence of Elp2. Adding recombinant, His-tagged Elp2 to the reactions did not

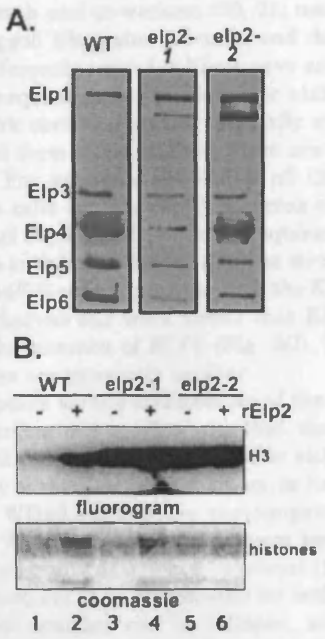

FIG. 5. **Five-subunit *elp2* Elongator complex retains HAT activity *in vitro*.** *A*, two independently purified *elp2* Elongator complexes and their subunit stoichiometry compared with wild type (*WT*). The Elp2 subunit is present only in wild type, but this Western blot is not shown. *B*, HAT activity of wild type and mutant Elongator complex with or without the addition of Elp2 as indicated. Please note that the activity of Elongator in HAT assays differs significantly from preparation to preparation and with freeze-thawing. It is therefore not possible to make a definitive judgment on the possible minor quantitative differences in activity, if any, between wild type and mutant Elongator.

dramatically affect the activity (the apparent stimulation of wild type complex (Fig. 5*B*, *lanes 1* and *2*) was because of loading differences). These data demonstrate that the WD40 repeat protein Elp2 is not required for Elongator HAT activity *in vitro*.

Elp3, but Not Elp2 or Elp4, Is Required for RNA Binding in Vivo—We have recently shown that the Elongator complex binds to RNA *in vitro* and that it is in close vicinity of nascent pre-mRNA *in vivo* (12). These results indicate that Elongator is associated with active genes in living cells. To investigate the subunit requirements for RNA association *in vivo*, we compared Elongator RNA-immunoprecipitation in wild type, *elp2*, and *elp4* cells expressing myc-tagged Elp3 (Fig. 6*A*). As shown previously (12), efficient cross-linking of Elp3 protein to *GAL1* mRNA was observed in wild type cells (Fig. 6*A*, compare *Elp3-myc* with *no tag*). Interestingly, although the levels were somewhat reduced, RNA interaction was also observed in cells lacking Elp2 (a component of core Elongator) and Elp4 (a core component of the small Elongator subcomplex). This indicates that neither the small subcomplex, nor Elp2 play crucial roles in the association of Elongator (Elp3) with transcripts *in vivo*. Because Elp3 levels are dramatically reduced in cells lacking Elp1, we could not investigate whether Elp1 is required for Elongator-RNA interaction. However, by tagging Elp1 rather than Elp3, the importance of the Elp3 protein for Elongator-RNA interaction could be investigated (Fig. 6*B*). Remarkably, Elongator-RNA interaction was ~5-fold reduced in the absence of Elp3, and this dramatic decrease was not because of changes in the expression level of the Elp1 protein (Fig. 6*B*, *bottom panel*). Taken together, these data indicate that the Elp3 protein plays a crucial role for the association of Elongator with active genes.

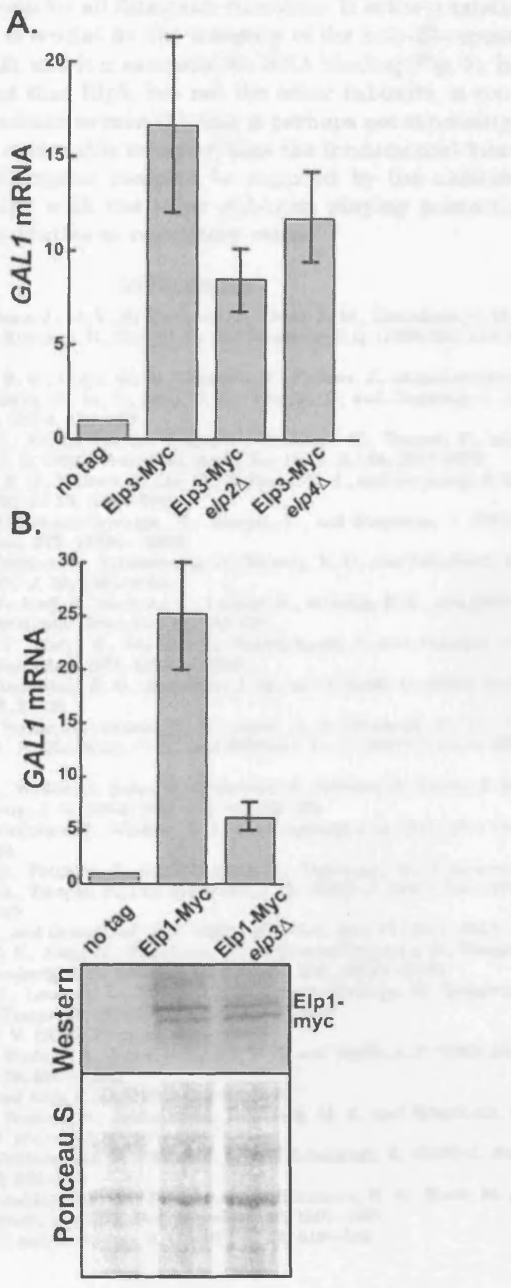

FIG. 6. **Elp3, but not Elp2 and Elp4, is required for Elongator-RNA association *in vivo*.** RNA-immunoprecipitation experiments in strains expressing myc-tagged Elongator subunits. *GAL1* mRNA was quantitated using reverse transcriptase real time PCR. *Bars* represent the average of two independent experiments. *Error bars* indicate variance. *A*, RNA-immunoprecipitation experiments with the indicated strains expressing Elp3-myc or the untagged control. *B*, RNA-immunoprecipitation experiments with the indicated strains expressing Elp1-myc or the untagged control. *Bottom panel* shows the result of an Elp1-myc Western blot with loading control (Ponceau S-stained membrane section) to demonstrate that Elp1 is stable in *elp3* cells. The resolution of Elp1 into two distinct bands has previously been described (22).

DISCUSSION

The results presented here represent the first comprehensive investigation of the structure-function relationship of Elongator, a histone acetyltransferase complex with a role in RNAPII transcription. Our results reveal the detailed molecular architecture of the complex and suggest roles, or unexpected lack of roles, for individual subunits.

Previous work (13–15) showed that holo-Elongator is composed of two weakly associated subcomplexes. Subsequent

work by Schaffrath and co-workers (20, 21) using immunoprecipitation of tagged Elongator subunits and detection of a co-precipitated differently tagged subunit gave some information about subunit requirements for Elongator stability. Although the present work confirms and significantly extends the conclusions derived from these studies, there are also surprising contradictions. For example, Frohloff *et al.* (21) detected the Elp3 protein in cells lacking *ELP1*, whereas our results indicate that normal Elp3 stability *in vivo* requires *ELP1* (Fig. 1). Likewise, these authors concluded that the structural integrity of the small Elp4/5/6 subcomplex requires the *ELP4*, *ELP5*, and *ELP6* genes, whereas our work shows that Elp4 can interact with Elp6 in the absence of *ELP5* (Fig. 2*C*). The reasons for these differences are presently unclear.

One of the theses for the architecture of the Elongator complex before starting our studies was that the WD40 repeat-containing Elp2 protein would turn out to either be essential for the integrity of the Elongator complex, or be required for its HAT activity. WD40 repeats are protein-protein interaction domains, and WD40-repeat proteins have been identified in other histone modifying/interacting proteins (18, 19). Surprisingly, Elp2 turned out to be dispensable for both; a five-subunit *elp2*Δ Elongator complex can be isolated, and this complex retains the ability to acetylate histones (Fig. 5). Elongator complex is also capable of binding RNA in the absence of *ELP2* (Fig. 6). Data from Schaffrath and co-workers (20) suggests that the role of Elp2 might be to allow interactions with other proteins, such as Kti12/Tot4. The Elongator-interacting protein, Kti12/Tot4 has been proposed to bridge interactions between RNAPII and Elongator. For example, the protein was shown to co-immunoprecipitate promoter DNA (chromatin immunoprecipitation) from the *ADH1* gene and to also be able to co-immunoprecipitate hyperphosphorylated RNAPII (20, 21). These authors also reported co-immunoprecipitation of hyperphosphorylated RNAPII with Elongator from the DNA-free soluble fraction of a yeast whole cell extract (21). For unknown reasons, we have so far not been able to reproduce these co-immunoprecipitation results, although we can detect Elongator-Kti12 interaction.[3] We therefore presently favor the idea that besides the hyperphosphorylated C-terminal domain, RNA in the ternary complex might play an important stabilizing role for the Elongator-RNAPII interaction (12).

In contrast to the Elp2 protein, the Elp3 subunit appears to play a crucial role for all Elongator functions. It is the catalytic subunit (2), it is crucial for the integrity of the holo-Elongator complex (Fig. 2), and it is essential for RNA binding (Fig. 6). In light of the fact that Elp3, but not the other subunits, is conserved from Archaea to man (2), this is perhaps not surprising. It thus seems reasonable to expect that the fundamental functions of the Elongator complex be supplied by the abilities intrinsic to Elp3 with the other subunits playing primarily function/augmentative or regulatory roles.

[3] T. G. Petrakis and J. Q. Svejstrup, unpublished data.

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